AMINO ACID SEQUENCE STUDIES OF LYSYL OXIDASE AND TRAMP (TYROSINE RICH ACIDIC MATRIX PROTEIN)

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Declaration of originality

I declare that, unless otherwise stated, this thesis represents my own work and was composed by me.

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Abstract

Lysyl oxidase initiates cross-linking in collagens and elastin by the conversion of specific lysine (and, in collagen, hydroxylysine) residues to peptidyl α-aminoadipic-δ-semialdehyde. Porcine skin lysyl oxidase (M_r = 32-34K) and a protein (M_r = 24K) with which the enzyme co-purifies have been isolated and characterised. The 24K protein was originally thought to be a degradation product of lysyl oxidase but this study shows it to be a distinct protein. Four variants of lysyl oxidase and five variants of the 24K protein were identified by Mono Q anion exchange Fast Protein Liquid Chromatography (FPLC). Each lysyl oxidase variant was subjected to amino acid analysis, which did not reveal any differences between variants, and to mass analysis, which showed small incremental changes between the variants. All the 24K protein variants were also indistinguishable by amino acid analysis, though the protein was clearly distinct from lysyl oxidase. As with lysyl oxidase, however, mass analysis showed small incremental changes between each 24K variant. Both lysyl oxidase and the 24K protein were found to be N-terminally blocked. A variety of cleavage methods was employed and the resulting peptides were subjected to mass and sequence analysis. Lysyl oxidase was cleaved using cyanogen bromide, and the N-terminal fragment was found and sub-digested with endoproteinase-Asp-N. The peptides formed were sequenced and from these studies it was possible to suggest the N-terminus of lysyl oxidase. The 24K protein was cleaved with cyanogen bromide and digested with various enzymes which included pyroglutamate aminopeptidase, clostripain, protease V8, and endoproteinase-Asp-N. The 24K protein was found to be unrelated to lysyl oxidase, but comparison with a protein sequence database showed it to be the same as a recently described protein from bovine skin that is associated with dermatan sulphate proteoglycans. The complete amino acid sequence of the 24K protein was determined and found to be relatively rich in tyrosine. Sequence analysis and chromatofocussing, using a Mono P chromatofocussing column, showed the protein to be acidic. In view of these properties the name TRAMP (Tyrosine Rich Acidic Matrix Protein) was proposed to identify the 24K protein. Though TRAMP appears not to be glycosylated, several experiments indicated the presence of sulphotyrosine residues. When assayed using an elastin substrate, the activity of lysyl oxidase was unaffected by TRAMP.

Contents

List of Figures

List of Tables

Abbreviations

The following is a list of abbreviations which are used in the main text of this report.

Amino acids

Single letter code	Three letter code	Amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
	hs	Homoserine
	hsl	Homoserine lactone
	Hyl	Hydroxylysine
	Hyp	Hydroxyproline
X		any amino acid, not known

Other abbreviations

A_{280}	-	absorbance at 280 nm
AA	-	amino acid
ABI	-	Applied Biosystems Incorporated
α-chain	-	individual polypeptide chain of collagen
ATZ	-	anilinothiazolinone
aq	-	aqueous
ßAPN	-	ß-aminopropionitrile
BCA	-	bicinchoninic acid
BDH	-	British Drug Houses Ltd.
Bis-Tris	-	Bis(2-hydroxyethyl)imminotris(hydroxymethyl)-methane
bp	-	base pair(s)
BRL	-	Bethesda Research Laboratories
BSA	-	bovine serum albumin
CAPS	-	3-[cyclohexylamino]-2-hydroxy-1-propane sulphonic acid
cDNA	-	complementary DNA
CF	-	Chou-Fasman
c.p.m.	-	counts per minute
C-propeptide	-	carboxy-terminal domain of procollagen
DEAE	-	diethylaminoethyl
DMEM		Dulbecco's modified Eagle's medium
DMPTU	-	dimethylphenylthiourea
DMSO	-	dimethylsulphoxide
d.p.m.	-	disintegrations per minute
DPTU	-	diphenylthiourea
DTT	-	dithiothreitol
EDTA	-	ethylenediaminetetraacetic acid
EPTU	-	ethylphenylthiourea
GAG	-	glycosaminoglycan
galN	-	galactosamine
glcN	-	glucosamine

GOR	-	Garnier, Osguthorpe and Robson
h	-	hour(s)
^3H	-	tritium
HVPE	-	high voltage paper electrophoresis
K	-	kilodaltons
KD	-	Kyte-Doolittle
kDa	-	kilodaltons
LDMS	-	laser desorption mass spectrometry
M	-	molar
MEM	-	modified Eagle's medium
µg	-	microgram(s)
mg	-	milligram(s)
min	-	minute(s)
ml	-	millilitre(s)
mM	-	millimolar
M_r	-	relative molecular mass
ND	-	not determined
NEM	-	N-ethylmalemide
nm	-	nanometer(s)
nmol	-	nanomole(s)
N-propeptide	-	amino-terminal propeptide of type I procollagen
PAGE	-	polyacrylamide gel electrophoresis
PB	-	phosphate buffer
PBS	-	phosphate buffered saline
PBU	-	phosphate buffered urea
PDMS	-	plasma desorption mass spectrometry
PE-C	-	pyridylethylcysteine
pI	-	isoelectric point
PIS	-	pre-immune serum
PITC	-	phenylisothiocyanate
pmol	-	picomole(s)
PMSF	-	phenylmethylsulphonylfluoride
PPO	-	2,5-diphenyloxazole
PQQ	-	pyrroloquinoline quinone

PTC	-	phenylthiocarbamyl
PTH	-	phenylthiohydantoin
PTU	-	phenylthiourea
proα1	-	α1 chain of procollagen
proα2	-	α2 chain of procollagen
SDS	-	sodium dodecyl sulphate
Staph.A.	-	Staphylococcus aureus
TEMED	-	N,N,N',N',-tetramethylenediamine
TFA	-	trifluoroacetic acid
TFMS	-	trifluoromethanesulphonic acid
TMA	-	trimethylamine
TRAMP	-	tyrosine rich acidic matrix protein
TRIS	-	Tris (hydroxymethyl) aminomethane
V	-	volt(s)
v/v	-	volume per volume

Chapter 1 Introduction

1.1 Introduction

Lysyl oxidase initiates cross-linking in collagens and elastin. A description of different collagen types and elastin is given in order to understand the role of lysyl oxidase. The function of TRAMP is not fully understood, and it is for this reason that details of other extracellular matrix proteins are provided to allow comparisons to be made between protein size, structure and function. Lysyl oxidase is then reviewed in detail followed by a description of TRAMP.

1.2 The extracellular matrix

Connective tissues include skin, tendon, blood vessels, cartilage and bone. They are systems of insoluble fibres and soluble polymers which have evolved to provide mechanical support in higher animals. All connective tissues consist of a number of matrix molecules, almost all of which are involved in interactions with other matrix components to form a composite material. Skin, tendon and blood vessels contain a range of different collagen types, elastin, proteoglycans and glycoproteins. Major constituents of cartilage are collagen fibres and proteoglycans. In bone the matrix is mineralised, and much of the hydroxyapatite mineral occurs within the collagen fibrils, while other matrix components may regulate the timing of mineral deposition and growth of crystals. Much of the assembly of the matrix is extracellular and a variety of components, and cell-matrix interactions, are involved in the assembly process. Different components of the extracellular matrix are discussed below.

1.3 Collagens

1.3.1 Introduction

Collagens are a large group of insoluble fibrous proteins which have

high tensile strength (reviewed by Mayne and Burgeson, 1987; van der Rest and Garrone, 1991; Hulmes, 1992). They form the major fibrous elements in skin, tendon, blood vessels, cartilage, bone and teeth, and are the most abundant proteins in mammals where they form about 25% of total body dry weight. Collagens are present in most organs and serve to hold cells together in units. This wide ranging variety of function is reflected in the diversity of collagen structure.

The name collagen is derived from the French 19th century word collagene used to describe the constituent of connective tissue that produces glue, or gelatin on boiling. At this time early histologists discovered fibres in sections of connective tissue. In the 1920s it was discovered that collagen could be solublised in acid and precipitated as collagen fibres. In 1956 the existence of the monomeric unit of collagen (tropocollagen; reviewed by Gross, 1956) was found but today it is known as the collagen molecule.

1.3.2 Collagen structure

A collagen is defined as a structural protein of the extracellular matrix which contains one or more domains containing three polypeptide chains, which may or may not be identical, which combine together to form a triple helix. The primary structure of each polypeptide chain contains at least one region of the repeating amino acid sequence $(Gly\text{-}X\text{-}Y)_n$ where X and Y can be any amino acid. It is a characteristic feature of collagens that they have a high imino acid (proline and hydroxyproline) content and that they contain hydroxylysine, a post-translational modification rarely found in other proteins (van der Rest and Garrone, 1991).

Different collagens are classified into types using Roman numerals to indicate the chronological order of their discovery (Table 1.1). Collagen I was the first collagen type to be isolated, primarily from skin and tendon. The molecule has two identical $\alpha 1$ chains and one distinct $\alpha 2$ chain. Today the nomenclature $[\alpha 1(I)]_2\alpha 2(I)$ is used to describe collagen I. In 1969 collagen II was isolated from chick cartilage and found to be a homotrimer with the structure $[\alpha 1(II)]_3$ (Miller and Matukas, 1969). Shortly afterwards collagen III was isolated from foetal skin and found to be a homotrimer with the structure

Table 1.1. Vertebrate collagens

Type	α-chains	Most common molecular form	Representative tissues
Fibrillar collagens			
I	α1(I), α2(I)	$[\alpha1(I)]_2\alpha2(I)$	skin, tendon and bone
II	α1(II)	$[\alpha1(II)]_3$	cartilage and vitreous humour
III	α1(III)	$[\alpha1(III)]_3$	skin, lung and vascular system
V	α1(V), α2(V), α1(V)	$[\alpha1(V)]_2\alpha2(V)$	collagen I containing tissues
XI	α1(XI), α2(XI) α3(XI)	α(XI)α2(XI)α3(XI)	collagen II containing tissues
Nonfibrillar collagens			
IV	α1(IV), α2(IV) α3(IV), α4(IV) α5(IV)	$[\alpha1(IV)]_2$ α2(IV)	basement membranes
VI	α1(VI), α2(VI) α3(VI)	α1(VI)α2(VI)α3(VI)	skin and cartillage
VII	α1(VII)	$[\alpha1(VII)]_3$	dermal-epidermal junction
VIII	α1(VIII), α2(VIII)	$[\alpha1(VIII)]_3$?	Descement's membrane
IX	α1(IX), α(IX) α3(IX)	α1(IX)α2(IX)α3(IX)	cartilage and vitreous humour
X	α1(X)	$[\alpha1(X)]_3$	hypertrophic zone of cartilage
XII	α1(XII)	$[\alpha1(XII)]_3$	tendon and skin
XIII	α(XIII)	$[\alpha1(XIII)]_3$?	skin and intestine
XIV	α1(XIV)	$[\alpha1(XIV)]_3$?	collagen I containing tissues
XV	α1(XV)	$[\alpha1(XV)]_3$?	not determined
XVI	α1(XVI)	$[\alpha1(XVI]_3$?	not determined

$[\alpha1(III)]_3$ (Miller *et al.*, 1971). Collagen III is similar to collagens I and II but contains disulphide bonds. In the early 1970s collagen IV was isolated from basement membranes. Its amino acid composition and size were similar to collagens I, II and III (Kefalides, 1973). However later work showed that collagen IV formed a meshwork of filaments in different planes rather than classical linear fibrils (Yurchenco and Schittny, 1990). The most common form of collagen IV, has the structure $[\alpha1(IV)]_2\alpha2(IV)$.

At present there are 16 recognised vertebrate collagen types (Hulmes, 1992; Pan *et al.*, 1992). In addition there are proteins with collagen-like features that are not called collagens. Such proteins contain $(Gly\text{-}X\text{-}Y)_n$ sequences, e.g. the complement component Clq (Sellar *et al.*, 1991).

Vertebrate collagens can also be classified according to their size and structure (Mayne and Burgeson, 1987; van der Rest and Garrone, 1991; Hulmes, 1992). There are three main groups. The fibrillar (D-staggered) collagens (I, II, III, V, and XI) form fibrils with a rod-like structure (diameter 20-500 nm) and characteristic 64-67 nm (distance D) repeating banding pattern as seen with the electron microscope. The non-fibrillar collagens (IV, VII, VIII, IX, X, XII, XIII and XIV) can be classified according to their structure and function. They can be subdivided into basement membrane collagens, short chain collagens and Fibril-Associated Collagens with Interupted Triple helices (FACIT collagens). Collagen VI forms fibrils but these are distinct from classical collagen fibrils as they do not have a D-periodic banding pattern.

FACIT collagens (IX, XII and XIV) are found attached specifically to the surface of fibrillar collagen fibrils. Collagen IX was the first to be discovered and was found to be associated with collagen II (van der Rest and Mayne, 1988). Collagens XII and XIV have since been discovered and found to be associated with collagen I (Shaw and Olsen, 1991). Collagen IX is unusual as it carries a glycosaminoglycan (GAG) chain, although a form of collagen IX has been described which lacks a GAG chain (Ayad *et al.*, 1991).

Most collagen fibres are composed of two or more different types. In cartilage, the fibrils contain collagens II, IX, and XI, while skin fibrils contain collagens I and III.

1.3.3 Collagen biosynthesis

All fibrillar collagens (I, II, III, V and XI) are initially synthesised in precursor form, as procollagens, in fibroblasts, chondrocytes or smooth muscle cells. Most information on collagen biosynthesis comes from studies on procollagen I (Byers, 1990; Kuivaniemi *et al.*, 1991) which probably acts as a model for the rest of the collagen family.

Procollagen I consists of two α1 chains and one α2 chain, each consisting of about 1500 amino acids (M_r ~ 150K). Globular domains are located at both amino (N-) and carboxyl (C-) termini of the procollagen molecule straddling the (mainly) triple-helical collagen domain. The N-propeptide domain of each chain consists of about 150 amino acids (M_r ~ 15K) and the C-propeptide domain (each chain) is about 250 amino acids (M_r ~ 30K). The N-propeptides contain intra-chain disuphide links, whereas the C-propeptides are linked by inter-chain disulphide bonds (Fessler *et al.*, 1985). Disulphide bonds are not normally found in the triple-helical region of the mature collagen molecule.

Procollagens are subject to a large number of post-translational modifications. Certain of these enzymic modifications can only take place before triple-helix formation. Procollagen mRNA is translated to form a preprocollagen chain, with a short hydrophobic sequence at the N-terminus, the signal peptide, that is involved in translocation into the Golgi apparatus (Palmiter *et al.*, 1979). After insertion into the cisternae of the rough endoplasmic reticulum, proα chains are subject to inter-chain disulphide cross-linking, prolyl 4-hydroxylation (and also prolyl 3-hydroxylation), lysyl hydroxylation, hydroxylysine O-linked glycosylation in the triple helical region and N-linked glycosylation in the C-propeptide region. The enzyme protein disulphide isomerase (PDI) is involved in the formation of these inter-chain cross-links. Hydroxyproline and hydroxylysine are formed by post-translational modification.

Following transport through the Golgi system, procollagen molecules are packaged into secretory vesicles, where they become aligned together side-by-side, and then secreted into the extracellular matrix, The propeptides are cleaved in the matrix, where fibril formation (Kuivaniemi *et al.*, 1991) and

cross-linking ensue (Seyedin and Rosen, 1990). The N-propeptides are removed by procollagen N-proteinase and the C-propeptides are removed by procollagen C-proteinase (Prockop and Kivirikko, 1984). Both these enzymes are extracellular, neutral, calcium-dependent metalloproteinases (Hojima *et al.*, 1989). Collagen cross-linking is described in Section 1.8.2.

1.4 Elastin

1.4.1 Introduction

Elastin is a connective tissue protein which confers the properties of extensibility and elastic recoil to tissues where it is found (reviewed by Gosline, 1976; reviewed by Foster, 1982; Mecham and Heuser, 1991). Tissues which are subject to frequent deformation, tension and pressure changes, such as blood vessel walls and elastic ligaments (e.g. ligamentum nuchae), contain high concentrations of elastin. Lower concentrations of elastin are found in the parenchyma of lungs, in skin, tendon, ear cartilage and pericardium.

The name elastin is one of a number of related terms (others include "elacin", "elastica", "collacin" and "collastin"), in common usage in the mid 19th century. In 1836 Eulenberg isolated elastin from vascular walls using boiling water, and in 1841 Henle identified elastica in the walls of blood vessels. In 1880 it was discovered that elastin could be solubilised and reversibly precipitated by adjusting the temperature up to 60°C (reviewed by Hass, 1939). The first amino acid analysis of elastin was described by Stein and Miller (1938).

1.4.2 Elastin structure

Elastic fibres are made up of both microfibrillar and amorphous components. The microfibril component consists of filaments (diameter ~ 10-20 nm), with a characteristiclly high, polar amino acid content and many disulphide cross-links. The microfibrils are composed of a number of

glycoproteins such as fibrillin (Sakai *et al.*, 1986), 31K microfibril-associated glycoprotein (Gibson *et al.*, 1986) and a 36K microfibril-associated glycoprotein (Kobayashi *et al.*, 1989). The amorphous component is composed of elastin, and is found in close association with the microfibril component. Elastin consists of 95% non-polar amino acid residues, predominantly valine, alanine, glycine and proline. Although rich in proline, elastin is very low in hydroxyproline, and it contains no hydroxylysine, tryptophan, cysteine or methionine (Partridge, 1962). Within the elastin fibre, the individual elastin polypeptide chains are covalently linked together by the elastin specific cross-links desmosine and isodesmosine which are derived from oxidation of lysyl residues by lysyl oxidase (Pinnel and Martin, 1968; Section 1.8.3). Elastin contains several short repeating peptide sequences, e.g. the pentapeptide VPGVG which occurs 11 times in one section of the molecule, the hexapeptide VAPGVG and the tetrapeptide VPGG. All three repeating sequences contain the dipeptide sequence PG which is important in the formation of the β-turn structure. The β-turn is a 10 atom ring with the C-O of residue 1 hydrogen bonded to the N-H of residue 4 and the dipeptide PG residues 2 and 3. It is thought that the repeating peptides of elastin are arranged in β-turns to form helical β-spirals (Arad and Goodman, 1990). The polypentapeptide is responsible for the elastic properties of elastin, while the hexapeptide is a more rigid structure with hydrogen bonding between repeats. Hydrophobic association of the hexapeptide in this region could result in aligning and interlocking of chains and there are 6-fold hexapeptides repeats near the cross-linking regions (Foster *et al.*, 1973). Within the cross-linking regions, lysine residues are interspaced with alanine rich sequences (Section 1.8.3).

1.4.3 Elastin biosynthesis

Elastin is synthesised and secreted in precursor form, tropoelastin, a soluble, single polypeptide chain (M_r ~ 70K; Sandberg *et al.*, 1969; Foster *et al.*, 1975). The initial mRNA translation product pre-tropoelastin, has an N-terminal signal peptide which is cleaved after translocation into the cysternae of the rough endoplasmic reticulum (Mecham, 1991), where the tropoelastin

chains are subject to hydroxylation of prolyl residues. In contrast to collagen biosynthesis, post-translational hydroxylation of proline residues appears to be relatively unimportant. Only about 8% of proline residues in tropoelastin or mature elastin become hydroxylated. Lack of hydroxyproline does not affect secretion of tropoelastin monomers (Uitto *et al.*, 1976; Kao *et al.*, 1982), or oxidation of lysyl residues (Mecham, 1991). However, over-hydroxylation may affect the ability of tropoelastin to form mature fibres (Barone *et al.*, 1985). Tropoelastin molecules are secreted into the extracellular matrix, where they become deposited on to microfibrils (Rosenbloom, 1987). At least one protein has been identified that is involved in delivery of elastin to the microfibrils. This is a 67K cell surface associated elastin binding protein which binds to the hydrophobic hexapeptide VGVAPG (Mecham *et al.*, 1989). Cross-linking of elastin is described in Section 1.8.3.

1.5 Proteoglycans

1.5.1 Introduction

Connective tissues are also rich in proteoglycans (reviewed by Heinegard and Sommarin, 1987; Wight *et al.*, 1991). Proteoglycans are found in the extracellular matrix, on cell surfaces, and in intracellular granules. The large proteoglycans are important in the visco-elastic properties of joints and other tissues which are subject to physical deformation. The small proteoglycans often interact specifically with collagens (Scott, 1988). Cell surface proteoglycans are believed to take part in cell substrate adhesion, cell recognition and growth regulation (Piepkorn *et al.,* 1990).

1.5.2 Proteoglycan structure

Proteoglycans are heavily glycosylated proteins with covalently attached glycosaminoglycan (GAG) chains (Lohmander, 1988). The GAGs are large polysaccharides composed of repeating disaccharide units. There are four main types of GAGs: heparan/heparan sulphate, chondroitin

sulphate/dermatan sulphate, keratan sulphate and hyaluronic acid. The first three GAGs are always protein bound and sulphated, while hyaluronic acid lacks sulphate and is synthesised as a free GAG chain.

GAGs, especially sulphated GAGs, are strongly negatively charged, enabling them to bind to many substances, for example many growth factors and cytokines bind to heparan sulphate proteoglycans. The binding of fibroblast growth factors (FGFs) to syndecan (Section 1.5.2.3) appears to protect the growth factor from degradation (Ruoslahti and Yamaguchi, 1991). At least one growth factor, transforming growth factor β (TGFβ) binds to proteoglycans via the core protein, as found with betaglycan (Andres *et al.*, 1989; Section 1.5.2.3).

In the absence of methodical nomenclature, the practice has arisen of identifying families of proteoglycans according to their protein cores. There are many different types of core protein, which vary in size and structure, and these are described below.

1.5.2.1 Aggrecan and versican

Aggrecan, found in articular cartilage, is a large proteoglycan (overall M_r ~ 10^6K, core protein M_r ~ 220K; Doege, 1991) which contains two types of GAG, keratan sulphate and chondroitin sulphate, and can form large aggregates with hyaluronic acid and link protein. In human articular cartilage the structure of the aggrecan molecules changes with increasing age (Bayliss, 1990). This is due to a decrease in the number and size of the chondroitin sulphate chains, and increase in the number and size of the keratan sulphate chains. Versican is a large, chondroitin sulphate proteoglycan expressed by human fibroblasts (protein core M_r ~ 260K; Zimmermann and Ruoslahti, 1989), which may be involved in cell recognition, possibly by connecting extracellular matrix components and cell surface glycoproteins.

1.5.2.2 Serglycin, biglycan, decorin, lumican and fibromodulin

Serglycin (core protein M_r ~ 20K), biglycan (core protein M_r = 38K),

decorin (core protein M_r = 36.5K), lumican (core protein M_r = 38.6K) and fibromodulin (core protein M_r ~ 59K) are members of a family of small proteoglycans. Serglycin is the smallest of this family and is named after the 24 consecutive serine-glycine repeats in its core protein (Bourdon *et al.*, 1985). It is an intracellular proteoglycan found in storage granules of hematopoetic cells and mast cells.

Biglycan, decorin, lumican and fibromodulin show homologous core protein structures but represent different gene products (Hedbom and Heinegard, 1989; Fisher *et al.*, 1991; Blochberger *et al.*, 1992). They each contain a central part with ten characteristic leucine-rich repeats and, close to the C-terminus, a loop formed by disulphide bonds. In the most N-terminal parts the four proteoglycans show less similar core protein structures and markedly different post-translational modifications.

Decorin, lumican and fibromodulin bind to collagens types I, II, VI, IV, and XI where binding is mediated by the protein core. These proteoglycans interfere with collagen fibrillogenesis and may be important in determining the surface properties of the completed fibril (Hedbom and Heinegard, 1989; Fleischmajer *et al.*, 1991). Collagen fibrils possess four sites that can bind proteoglycans via their protein cores (Scott, 1992). Proteoglycan-GAG chains can then aggregate to link adjacent fibrils, thereby preventing them from slipping and increasing the tensile strength. Biglycan binds only to collagen IV (Fisher *et al.*, 1991).

1.5.2.3 Syndecan-1, fibroglycan, syndecan-3, ampiglycan, glypican and betaglycan

Syndecans are a family of integral cell surface proteoglycans with conserved intracellular and transmembrane domains, but, apart from the GAG attachment regions, relatively variable extracellular domains. Syndecan-1 (protein core M_r = 85K; Saunders *et al.*, 1989) interacts selectively with extracellular matrix molecules, e.g. fibrillar collagens, fibronectin, thrombospondin and tenascin, via its heparan sulphate side chains. The binding of syndecan I to basic fibroblast growth factor (bFGF) suggests that cell surface proteoglycans can be low affinity receptors for growth factors.

Fibroglycan, also known as syndecan-2, is a transmembrane, cell surface heparan sulphate proteoglycan (core protein M_r = 48K; Pierce *et al.,* 1992). Syndecan-3 (Gould *et al.,* 1992) is an integral membrane proteoglycan identified from an embryonic chick wing bud cDNA library, which is structurally related to syndecan-1 and fibroglycan. Ampiglycan is yet another transmembrane, cell surface proteoglycan (core protein M_r = 35K; Lories *et al.,* 1992). Glypican is a cell surface heparan sulphate proteoglycan which differs from syndecan-1, fibroglycan, syndecan-3 and ampiglycan in that it is anchored to the membrane by a glycosylphosphatidylinositol (GPI) tail.

Betaglycan is a 250-300K cell surface proteoglycan (core protein M_r ~ 120K; Andres *et al.,* 1989) which carries chondroitin sulphate and/or heparan sulphate GAG chains. Transforming growth factors β1and β2 bind with high affinity to the core protein of this proteoglycan.

1.5.3 Proteoglycan biosynthesis

During proteoglycan biosynthesis, core protein mRNA is translated and newly synthesised chains are translocated into the rough endoplasmic reticulum (Hascall *et al.*, 1991). The heparan/heparan sulphate, chondroitin sulphate, dermatan sulphate and keratan sulphate become attached to the core protein in the Golgi apparatus. The completed monomer is then secreted via secretory vesicles into the extracellular matrix. In the case of aggrecan, it is believed that the link protein and hyaluronate are secreted separately, so that aggregation occurs in the extracellular matrix.

1.6 Structural glycoproteins

1.6.1 Introduction

Structural glycoproteins play an important role in many cell surface interactions (reviewed by Yamada, 1991). Each of these glycoproteins can participate in a range of functions using different specific domains, e.g. the RGD cell binding sequence, to bind to cell surface receptors or other

extracellular matrix macromolecules.

1.6.2 Fibronectin and vitronectin

Fibronectins are a family of matrix glycoproteins that enable cells to interact with the extracellular matrix (Ruoslahti *et al.*,1982). Fibronectin was first identified in plasma but has since been widely found. The fibronectin molecule is a dimer with two large polypeptide chains (M_r ~ 220K) which are linked by a disulphide bridge near their C-termini. In the centre of the molecule there is an RGD cell-binding site (Ruoslahti and Pierschbacher, 1986) which facilitates binding to cell surface integrin receptors. Other domains of fibronectin interact with glycosaminoglycans and collagen fibres (Kirchofer *et al.*, 1991).

Vitronectin is a glycoprotein (M_r = 75K) which, like fibronectin, promotes cell attachment and interacts with proteoglycans and GAGs. It also contains an RGD cell attachment site (Suzuki *et al.*, 1985).

1.6.3 Laminins and nidogen

Laminins are a family of large (unreduced M_r = 800-900K) multidomain glycoproteins with multiple functions in cellular processes and in the supramolecular assembly of basement membranes (Yurchenco and Schittny, 1990). Cellular functions attributed to laminin include promotion of growth differentiation, neurite growth, cell mobility and attachment, and mediation of cell communication (Hunter *et al.*, 1989). Classical laminin is the most abundant non-collagenous protein in basement membranes. It is composed of three polypeptide chains. The central A chain (M_r ~ 400K) is flanked by B1 and B2 chains (M_r ~ 200K). The three chains are disulphide bonded at two sites to form an asymmetrical four armed molecule.

Nidogen, also called entactin, is also a basement membrane glycoprotein (M_r = 148K; Fox *et al.*, 1991). The molecule consists of three globular domains, G1, G2 and G3, a short link region connecting G1 and G2 and a rod-like domain between G2 and G3. Nidogen binds to collagen IV at its G1 and G2 region and binds to laminin at its G3, C-terminal region. It is

believed to play a key role in the assembly of basement membranes.

1.6.4 Fibrillin, 31K and 36K microfibril associated glycoproteins and 34K structural glycoprotein

Fibrillin is a glycoprotein (M_r = 350K) that is associated with extracellular microfibrils (Sakai *et al.,* 1986). Fibrillin is widely found in connective tissues of skin, lung, cardiovascular system, tendon, muscle and cornea. It has recently been shown that mutations in the fibrillin gene are responsible for Marfan's syndrome (Section 1.8.11.1; Dietz *et al.*, 1991).

31K microfibril associated glycoprotein (31K MAP) is an acidic glycoprotein that is associated with elastin microfibrils and which has been isolated from bovine nuchal ligament (Gibson *et al.,* 1986). The function of 31K MAP is unknown. It is of similar size to lysyl oxidase (Section 1.8), and it has been suggested that 31K MAP may be an elastin-specific form of lysyl oxidase. However, this suggestion is not supported by the amino acid composition which, compared to bovine lysyl oxidase, is relatively rich in glutamate, proline, valine, leucine, lysine and cystine, and low in glycine and tyrosine. In addition anti-bovine lysyl oxidase antiserum shows no cross-reactivity with 31K MAP (Gibson *et al.,* 1986).

A 34K structural glycoprotein (SGP), containing 10% carbohydrate, has been extracted from bovine ligamentum nuchae, which exhibits peptidyl-lysyl oxidase activity (Serafini-Fracassini *et al.*, 1981). From its amino acid composition SGP appears to be quite distinct from both lysyl oxidase and 31K MAP. Compared to lysyl oxidase SGP is relatively rich in glycine, leucine and lysine residues and has a lower tyrosine content. As SGP exhibits amine oxidase activity towards free lysine as well as peptidyl-lysine, it would appear to belong to a different class of enzymes to lysyl oxidase.

36K microfibril associated glycoprotein (36K MAP) is an adhesive glycoprotein which has been isolated from porcine aorta and shown to contain an RGD cell binding sequence in its N-terminal region (Kobayashi *et al.,* 1989). It is found associated with elastin microfibrils and is distinct from the 31K microfibril associated glycoprotein and SGP.

1.6.5 Tenascins, thrombospondin, cartilage oligomeric matrix protein (COMP) and von Willebrand factor (vWF)

Tenascins are a family of large six armed extracellular matrix glycoproteins (unreduced M_r ~ 1,300K; Erickson and Inglesias, 1984; Vaughan *et al.,* 1987). Tenascin molecules are homohexamers with one arm corresponding to one subunit (M_r ~ 190-340K). Several tenascin variants have been described, which arise from alternative splicing, and all contain cell binding (RGD) and heparan binding sequences. Tenascin shows a very interesting tissue distribution as it appears in large amounts in a very restricted manner during embryonic development (e.g. extracellular regions of developing or regenerating tisue). In adult tissues tenacins can be found in tendons, ligaments, pericardium and periosteum (Nies *et al.*, 1991). Tenascins identified include tenascin, restrictin and tenascin-MHC (major histocompatability complex).

Thrombospondin is an adhesive glycoprotein (unreduced M_r ~ 600K, Gehron-Robey *et al.,* 1989). Endothelial cells can synthesise and incorporate newly synthesised thrombospondin into their extracellular matrix where it interacts with macromolecules such as fibronectin and collagen V. Thrombospondin is synthesised by different cell types, e.g. platelets, endothelial and smooth muscle cells, and the protein can modulate *in vitro* cell adhesion, spreading and mobility. Thrombospondin is composed of three identical disulphide-linked subunits (subunit M_r ~ 180K) and the molecule has a calcium-dependent conformation. As found in other extracellular matrix proteins, thrombospondin possesses several structural domains which exhibit different functions, including an RGD cell binding sequence.

Cartilage Oligomeric Matrix Protein (COMP) is a five-armed matrix protein isolated from articular cartilage (unreduced M_r ~ 500K; Morgelin *et al.*, 1992; Hedbom *et al.,* 1992), with a similar structure to thrombospondin. The function of COMP is unknown.

Von Willebrand factor (vWF) is a large oligomeric glycoprotein (M_r ~ 260K), synthesised by endothelial cells and deposited in their extracellular matrix (Sadler, 1991). vWF has two important functions in haemostasis. First, it is required for adhesion of platelets to sites of vascular damage and, second,

it binds to and stabilises blood coagulation factor VIII (antihaemophilic factor) in the circulation. vWF binds to fibrillar collagens, collagen type VI, heparin and sulphated glycolipids. An RGD sequence has been identified near its C-terminus.

1.6.6 Bone sialoprotein (BSP), osteopontin, secreted protein acidic and rich in cysteine (SPARC) and osteocalcin

Two human bone sialoproteins (BSP I and BSP II; M_r = 70-80K) have been isolated (Fisher *et al.*, 1987). They differ in their sialic acid content, their amino acid composition, and their phosphate content. BSP II has a core protein (M_r = 33.6K), which contains a RGD cell binding sequence and binds to cells via a vitronectin-type integrin receptor (Oldberg *et al.,* 1988a, b). BSP II also contains a repeating sequence motif of up to ten adjacent residues which may confer calcium binding properties to the protein, and the protein is tyrosine sulphated (Ecarot-Charrier *et al.*, 1989). The precise role of bone sialoproteins is unknown.

Osteopontin is a bone glycoprotein (M_r = 32.6K) that is similar to bone sialoprotein but with a lower carbohydrate content (Heinegard and Oldberg, 1989). Osteopontin also contains a RGD cell binding sequence, phosphorylated serines and a sequence of adjacent aspartic acid residues. Osteopontin may be involved in recruiting osteoclast precursor cells and binding them to the mineralised matrix of bone.

Secreted Protein Acidic and Rich in Cysteine (SPARC), also known as osteonectin and BM40, is a developmentally regulated, secreted, calcium binding glycoprotein (M_r = 33K), which is associated with cellular events requiring tissue remodelling, cell movement and/or proliferation. SPARC contains no RGD sequences (Heinegard and Oldberg, 1989; Lane and Sage, 1990).

Osteocalcin is a low molecular weight protein (M_r = 5.8K) found only in bone and teeth (Heinegard and Oldberg, 1989). The presence of three γ-carboxyglutamic acid residues per molecule confers calcium binding so it has been suggested that osteocalcin can act as an inhibitor of calcification.

1.7 Connective tissue degradation

There are several classes of proteinases involved in connective tissue degradation and these are described below.

1.7.1 Metalloproteinases

The metalloproteinases are a family of enzymes that can degrade most of the components of the extracellular matrix (Okada *et al.*, 1986). They can be subdivided into three major classes, collagenases, gelatinases and stromelysins. These potent enzymes are secreted in proenzyme form and require activation before the substrate can be digested. This can be achieved, at least *in vitro*, by proteinases such as trypsin or plasmin.

Once activated, collagenase activity can be inhibited by tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2; Osthues *et al.*, 1992). TIMP-1 is glycoprotein (M_r = 28.5K), while TIMP-2 is non-glycosylated (M_r = 21K). These proteins inhibit the activity of matrix metalloproteinases by forming a one to one complex with the enzyme.

There are several possible control points where the level of metalloproteinase activity could be regulated These include synthesis, secretion and activation of the enzyme, and production of inhibitors. Although a plethora of cytokines and growth factors have been shown to modulate levels of collagenase and TIMP, little is known concerning feedback mechanisms and controls (Bertraux *et al.,* 1991; Hayakawa *et al.*, 1992).

1.7.2 Other proteinases

Polymorphonuclear leucocyte elastase, a serine proteinase, can degrade various collagen types, collagen telopeptides, proteoglycans as well as elastin (Rosenbloom, 1984). Macrophage elastase can degrade elastin and collagen IV. During various pathological processes, such as emphysema or atherosclerosis, elastin is degraded by elastases. Soluble elastin peptides are then released into the blood stream. The complete amino acid sequence

of cathepsins B, D, H and L have been determined (Bechet *et al.*, 1991). These proteinases can degrade various collagen types, elastin and proteoglycans.

1.7.3 Regulation of matrix synthesis and degradation

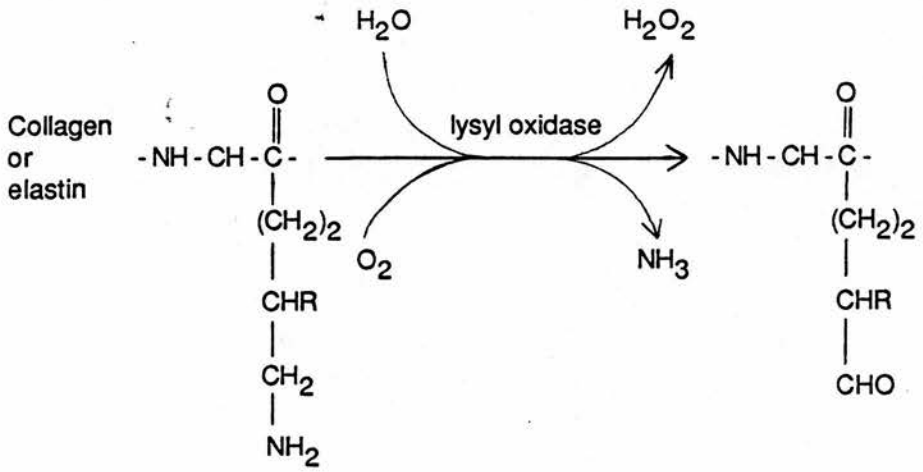
A number of mediators have been found which regulate production of matrix components. Growth factors, e.g. fibroblast growth factors (FGFs) and transforming growth factor β (TGFβ), increase the rate of proliferation of fibroblasts, chondrocytes and osteoblasts (Rifkin and Moscatelli, 1989). As the synthesis of collagen, proteoglycans and GAGs increases, the secretion of metalloproteinases decreases. Cytokines, e.g. interleukin-1, have an antagonistic effect. They decrease the synthesis of collagen, proteoglycans and GAGs and increase the release of collagenase (Redini *et al.,* 1988).

1.8 Lysyl oxidase

1.8.1 Introduction

Lysyl oxidase (protein-lysine-6-oxidase, E.C.1.4.3.13.) initiates cross-linking in collagens and elastin by the extracellular conversion of specific lysine (and, in collagen, hydroxylysine) residues to α-aminoadipic-δ-semialdehyde (allysine, or hydroxyallysine; Fig. 1.1; Pinnel and Martin, 1968; reviewed by Siegel, 1979; reviewed by Kagan, 1986). Spontaneous condensation between these aldehyde groups, and other vicinal aldehydes and unmodified lysine (or hydroxylysine) residues leads to the formation of a variety of intra- and inter-molecular cross-links (Fig. 1.2). These reactions convert soluble precursor forms of collagen and elastin to insoluble fibres. The formation of cross-links enables these fibres to withstand tensile stress.

The existence of a cross-linking enzyme was implied from studies of lathyrism, a connective tissue disorder in which no cross-links are formed. Symptoms of lathyrism, which can be induced experimentally by supplying β-aminopropionitrile (βAPN) or depleting copper from the diet, include aortic



R = H, peptidyl lysine or
R = OH, hydroxylysine

peptidyl α-amino adipic-δ-semialdehyde
(allysine) or
α-amino adipic-δ-hydroxysemialdehyde
(hydroxyallysine)

Fig. 1.1. Enzymic reaction catalysed by lysyl oxidase

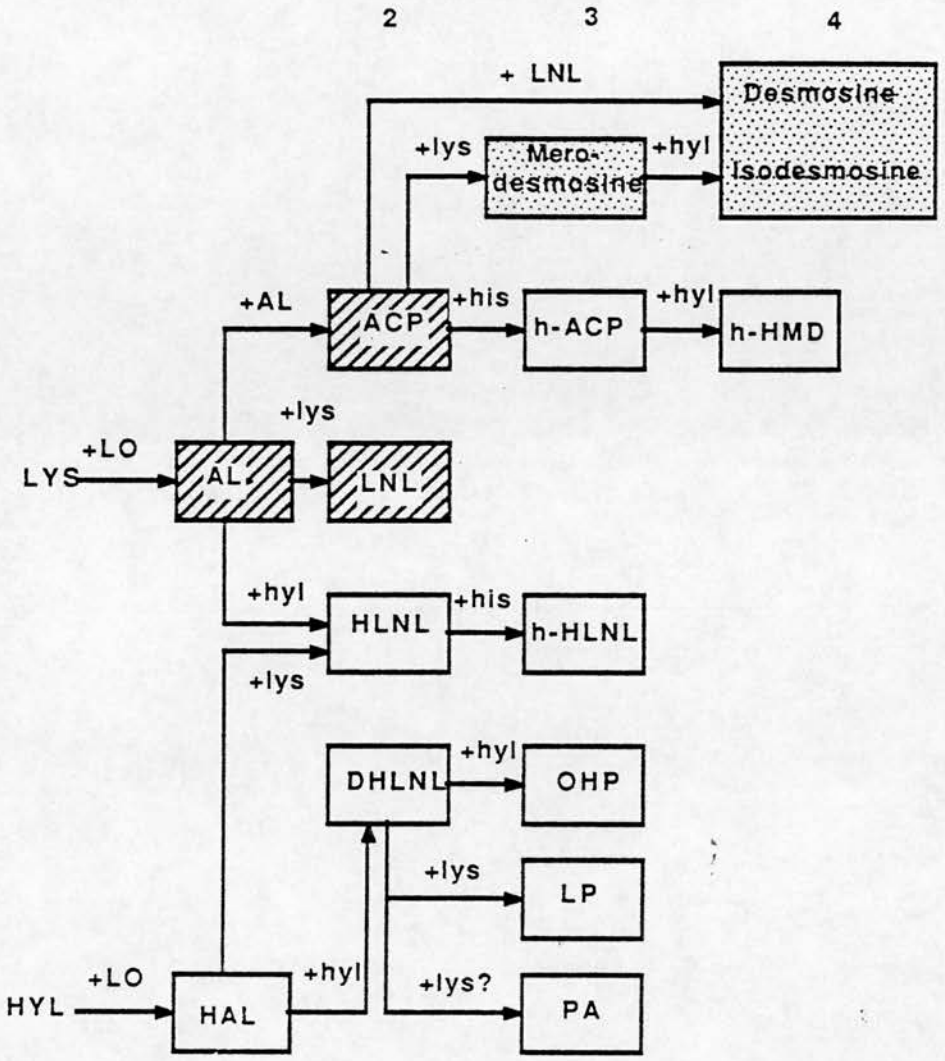


Fig. 1.2. Reactions of peptidyl lysine and hydroxylysine in the biosynthesis of cross-links in collagen and elastin

After oxidative deamination to form reactive aldehydes, subsequent condensation reactions lead to the formation of di-, tri-, and tetrafunctional cross-links. The number of residues involved in each cross-link are shown by the column headings 2, 3, and 4. Cross-links common to both collagen and elastin are shown in hatched boxes, cross-links specific to elastin are shown by the stippled boxes and cross-links specific to collagen are shown by open boxes. Abbreviations used are: LO (lysyl oxidase), AL (allysine), HAL (hydroxyallysine), ACP (aldol condensation products), LNL (lysinonorleucine), HLNL (hydroxylysinonorleucine), h-HLNL (histidine-HLNL), DHLNL (dihydroxylysinonorleucine), HMD (hydroxymerodesmosine), OHP (hydroxypyridinium), LP (lysylpyridinium), and PA (pyridinium analogue; from Reiser *et al.*, 1992).

rupture, skeletal deformities, and skin fragility. In 1968, lysyl oxidase was discovered and its activity shown to be inhibited by βAPN (Pinnel and Martin, 1968).

1.8.2 Cross-linking in collagen

Lysyl oxidase initiates cross-linking in fibrillar collagens by the oxidation of specific lysine, or hydroxylysine residues, located in the short N- and C-terminal, non-triple-helical telopeptide regions of the collagen molecule.

Cross-linking occurs in the extracellular matrix and requires specific alignment of collagen molecules (Fig. 1.3). Lysines, or hydroxylysines, in the N- and C-terminal telopeptide regions of each α chain become cross-linked to triple helical regions of neighbouring molecules within the collagen fibrils. The structures of some typical difunctional cross-links are shown in Fig. 1.4. Glycosylated as well as hydroxylated residues may also play a part in collagen cross-linking (Section 1.8.11.3; Kagan, 1986; Reiser *et al.*, 1992).

1.8.3 Cross-linking in elastin

The same lysyl oxidase enzyme appears to be involved in both collagen and elastin cross-linking (Siegel, 1979). Cross-link formation in elastin follows the same course as in collagen but with three major exceptions. Hydroxylysine is not involved as it is absent in elastin; histidine is also not involved; and the final, tetrafunctional cross-links, desmosine and isodemosine, are not found in collagens (Figs. 1.2 and 1.5).

Tropoelastin isolated from copper-deficient pig aortas contains approximately 47 lysine residues per molecule, while mature elastin contains approximately five lysine residues per molecule (Sandberg *et al.*, 1981). This decrease in the number of free lysine residues is explained by the involvement of the remaining lysines in lysyl oxidase initiated cross-links. This relatively large number of cross-linked lysines in elastin contrasts with the limited number of lysines oxidised per α chain of fibrillar collagen.

Lysine residues in tropoelastin are generally found in pairs and

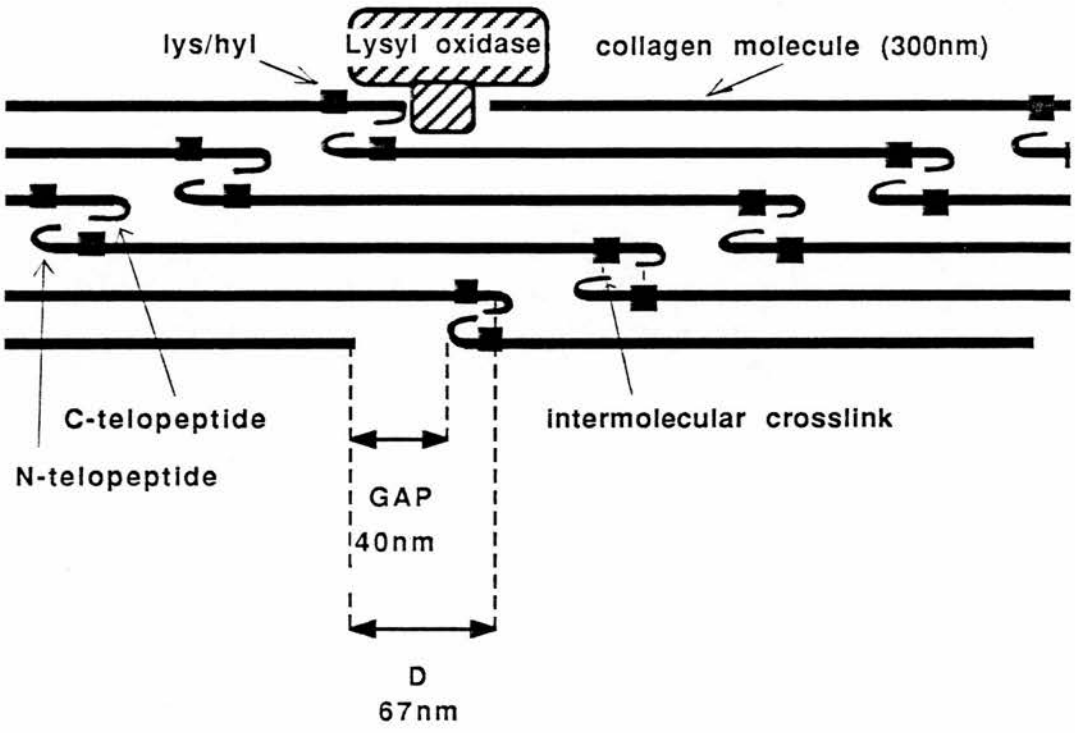
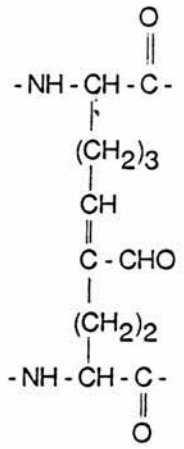
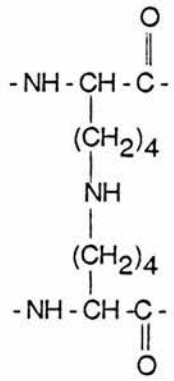


Fig. 1.3. Lysyl oxidase and fibrillogenesis of collagen I



Aldol condensation product (ACP)



Lysinonorleucine (LNL)

Fig. 1.4. Structures of difunctional cross-links

Aldol condensation product (ACP, formed from two adjacent allysine residues) is an intramolecular cross-link, while lysinonorleucine (LNL) is the reduced form of the intermolecular cross-link formed by condensation of lysine and allysine.

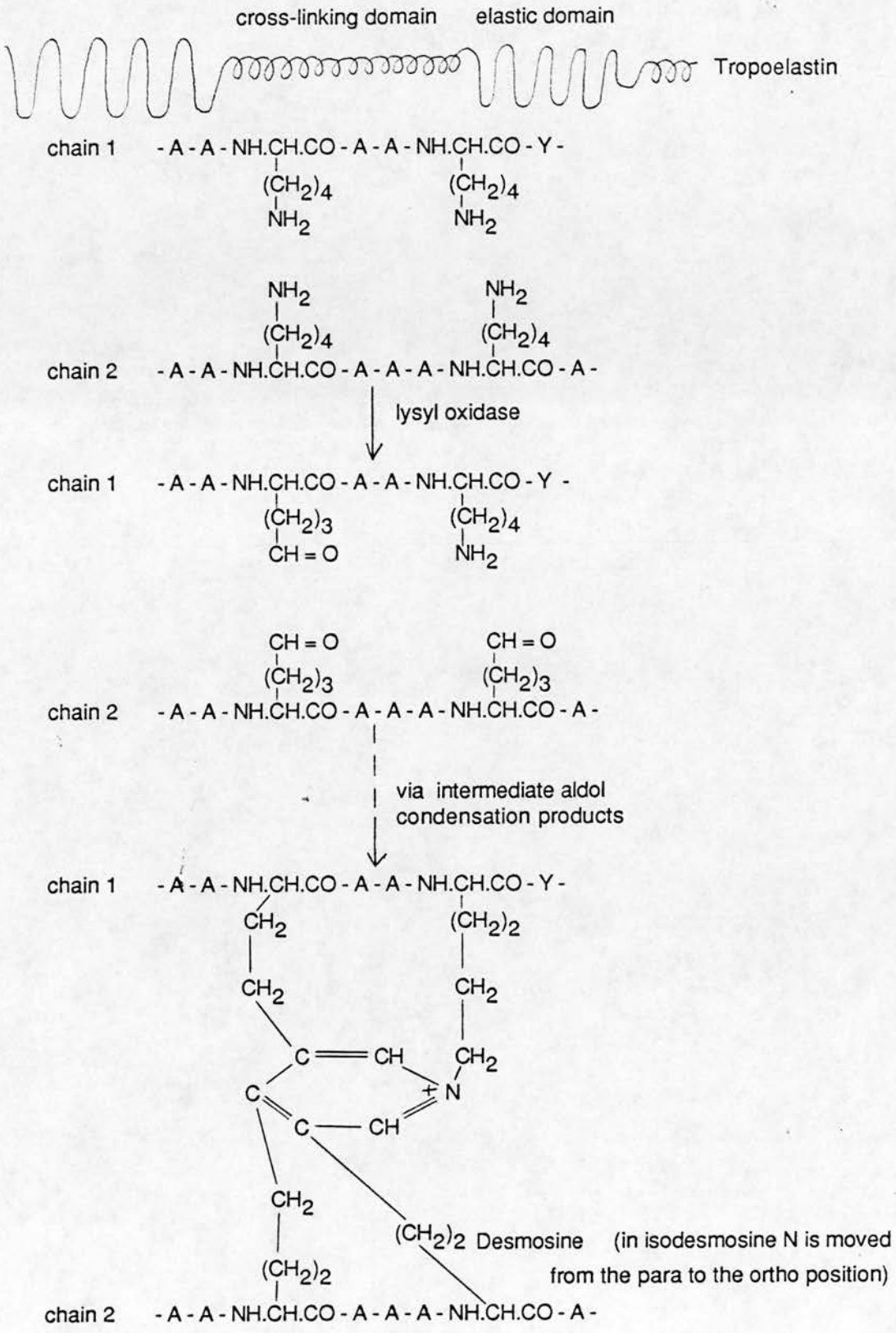


Fig. 1.5. Cross-linking of soluble elastin into insoluble elastin

(adapted from Sandberg *et al.*, 1981)

separated by one to three alanine residues. Preceding such sequences are as many as eight alanine residues in continuous sequence. This feature of alanine enrichment is unique to elastin (24% of total amino acids are alanine) and essential for cross-link formation.

Cross-linking in elastin involves oxidation, by lysyl oxidase, of three out of four lysine side chains in two adjacent chains (Fig. 1.5). Two difunctional cross-links, dihydrolysinonorleucine (DHLNL; formed from one residue of allysine and one lysine) and aldol condensation product (ACP; formed from two residues of allysine) condense to form desmosine or isodesmosine, thereby linking two chains with tetrafunctional cross-links (Fig. 1.5; Reiser *et al.*, 1992).

1.8.4 Molecular weight and enzyme variants

Molecular weight values for lysyl oxidase have largely been determined by SDS-PAGE analysis. Lysyl oxidase has been purified from a variety of tissues and from a number of organisms (Table 1.2), and there is a wide diversity in the reported molecular weights. The highest molecular weights (170-180K) are from salt extracts of bovine aorta (Shieh *et al.*, 1975), and chick cartilage (Siegel *et al.*, 1970b). In contrast, when urea is used in the extraction of the enzyme, the apparent molecular weight is in the range 28-34K, with 32K being the most frequently reported value (Kagan *et al.*, 1979). Lysyl oxidase is known to form high molecular weight aggregates in the absence of urea (Kagan *et al.*, 1979).

A number of procedures have been used to purify lysyl oxidase from the initial extracts. These include DEAE ion exchange chromatography, gel filtration chromatography, and a variety of affinity binding steps (to collagen, elastin, etc.; Table 1.2). When a DEAE column is the first stage in purification, molecular weight values of between 59-62K have been reported. This was found to be the case for bovine aorta (Vidal *et al.*, 1975), chick aorta (Harris *et al.*, 1974), and chick cartilage (Siegel and Fu, 1976). In certain cases it was noted that the 32K protein slowly degrades *in vitro* into a 24K protein and other products (Sullivan and Kagan, 1982). The human placental lysyl oxidase (M_r = 30K) was found to co-purify with a 24K protein, and this was

Table 1.2. Review of methods for purifying lysyl oxidase

Source	Reference	Purification	Variants	M_r
1.Bovine				
aorta	Shieh *et al.*,1975	PBS,ASP,DE,HA,BG,AF-ConA	2	180 (BG)
	Vidal *et al.*,1975	U,DE,AF-OCMP	2	59,61
	Jordan *et al.*,1977	U,AF-Col,DE,S-200	3	~30 (SDS)
	Kagan *et al.*,1979	U,AF-Col,DE,S-200	4	32 (SDS)
	Tang *et al.*,1983	U,AF-Col,DE,S-200		32 (SDS)
	Gacheru *et al.*,1990	U,CM-T,DE-T,AF-CB,S-200		
	Trackman *et al.*,1990	U,CM-T,DE-T,AF-CB,S-200		32
cartilage	Sulivan and Kagan,1982	U,AF-Col,DE,S-200		32 (SDS)
ligament	Jordan *et al.*,1977	U,AF-Col,DE,SX	4	~30 (SDS)
lung	Shieh and Yasunobu,1976	U,ASP,DE,AF-Col	2	80,160 (Native) 28,53 (SDS)
2.Calf				
aorta	Ferrera *et al.*,1982	U,AF,DE,E-H,SW-300		~30 (HPLC)
	Williams and Kagan,1985	U,CM-T,DE-T,AF-CB,S-200	4	32 (SDS)
cartilage	Han and Tanzer,1979	U,CM-T,DE-T,S-200	4	32 (SDS)
3.Chick				
aorta	Harris *et al.*,1974	U,DE,AF-OCMP	2	59,61 (SDS)
cartilage	Siegel *et al.*,1970b	PBS,ASP,PHP,BG		170 (Native)
	Narayanan *et al.*,1974	PBS,ASP,U,ASP,AF-Col		ND
	Siegel and Fu,1976	U,DE,AF-Col,DE	2	62 (SDS)
	Stassen,1976	U,AF-Col,DE,DE	4	28 (SDS)
4.Human				
placenta	Kuivaniemi *et al.*,1984	U,AF-Col,DE,S-200	4	30 (SDS)
umbilical	Burbelo *et al.*,1986	U,AF-CB,DE,S-200	4	30 (SDS)
- cord	Tang *et al.*,1989	U,AF-Gel,		32
	Wakasaki and Ooshima,1990b	U,AF-CB,DE,S-200		32
5.Porcine				
skin	Shackleton and Hulmes,1990a	U,DE,S-200		34 (SDS)
6.Rat				
liver	Wakasaki and Ooshima,1990a	U		32 (SDS) (48, precusor)
lung	Almassian *et al.*,1991	U,DE-T,HA,S-200		32 (SDS)
skin	Romero-Chapman *et al.*,1991	U,AF-El,S-200		32 (SDS) (40, precursor)
7.Tricho -derma sp.	Laugalene *et al.*,1990	HIC,BSC-80,AF		43 (SDS)
8.Turkey				
aorta	Narayanan *et al.*,1982	U,AF-Col,DE		100,77,52 + others (SDS)

Abbreviations are as follows: AF, affinity chromatography, AF-CB, Cybachrome Blue affinity chromatography, AF-Col, collagen-Sepharose affinity chromatography, AF-ConA, ConA affinity chromatography, AF-El, elastin affinity chromatography, AF-Gel, gelatin affinity chromatography, AF-OCMP, chromatography with chick embryo aorta organ culture media proteins coupled to Sepharose, ASP, ammonium sulphate precipitation, BG, chromatography with BioGel, BSC-80, HPLC with BSC-80, CM-T, chromatography with CM-Tris-acryl, DE, DEAE chromatography, DE-T, chromatography with DE-Tris acryl, EH, chromatography with elastin coupled to Hydrogel, HA, hydroxyappatite chromatography, HIC, hydrophobic interaction chromatography, HPLC, high pressure liquid chromatography, PBS, phosphate buffered saline, S-200, chromatography with Sephacryl S-200, SDS, SDS gel electrophoresis, SW-300, SX, chromatography with Sephadex, HPLC with TSK SW-300, U, 4M or 6M urea.

also believed to be a degradation product of lysyl oxidase (Kuivaniemi *et al.*, 1984).

Different variants of lysyl oxidase have been found by gradient elution anion exchange columns. Four variants have been isolated from bovine aorta (Kagan *et al.*, 1979; Cronlund *et al.*, 1985), bovine ligament (Jordan *et al.*, 1977), chick cartilage (Stassen, 1976), and human placenta (Kuivaniemi *et al.*, 1984), and two variants from bovine aorta (Vidal *et al.*, 1975) and chick cartilage (Siegel and Fu, 1976; Table 1.2).

1.8.5 Co-factors

Copper is essential for lysyl oxidase activity (Kagan, 1986; Iguchi and Sano, 1985; Gacheru *et al.*, 1990), and the enzyme from chick bone and bovine aorta contains 1g atom of copper per molecule (Kagan and Trackman, 1991). Recently, a putative copper binding site has been suggested in the predicted 32K lysyl oxidase sequence which is consistent with the probable location of the active site (Trackman *et al.*, 1990; Trackman *et al.*, 1991).

A second co-factor in lysyl oxidase has long been suspected and this was initially thought to be pyridoxal phosphate (PLP; Rucker and O'Dell, 1970; Bird and Levene, 1982), though the presence of PLP in the bovine aortic enzyme has since been disproved (Williamson *et al.*, 1986a). Raman spectroscopy of an active site peptide of calf aorta lysyl oxidase is consistent with the presence of a pyrroloquinoline quinone (PQQ, or methoxatin) co-factor (Williamson *et al.*, 1986b), and the presence of PQQ has also been reported in a proteolytic fragment of human placental lysyl oxidase (van de Meer and Duine, 1986). The identification of PQQ in mammalian enzymes is difficult, however, as mg amounts of pure enzyme are required (Paz *et al.*, 1989). Also harsh extraction conditions may create PQQ artificially (McIntire, 1992). When rats were fed on a PQQ deficient diet, however, they showed lathyritic symptoms and had lower levels of both lysyl oxidase protein and activity, and these effects were reversed by a PQQ supplementation (Killgore *et al.*, 1989).

Recently, it has been suggested that topa quinone (6-hydroxydopa quinone) may be the co-factor in lysyl oxidase (Janes *et al.*, 1990; Thomson,

1991; Duine, 1991). Topa quinone is a contiguous part of the polypeptide chain in the form of a modified tyrosyl residue. Lysyl oxidase, however, does not posess the consensus sequence for this co-factor (Mu *et al.*, 1992; Janes *et al.,* 1992; Section 4.3.1).

1.8.6 Kinetics and mechanism of action

The enzyme kinetics of lysyl oxidase are complex (Gacheru *et al.*, 1990; Kagan and Trackman, 1991). At low substrate concentrations, activity does not follow Michaelis-Menton kinetics, perhaps because lysyl oxidase has both catalytic and non-catalytic binding sites for elastin and collagen substrates, by analogy with myosin I (Lynch *et al.*, 1986).

Lysyl oxidase appears to follow a ping pong mechanism with an ordered substrate binding and product release. The proposed mechanism is as follows. The amine substrate is initially oxidised to the aldehyde by passage of two electrons from the α-carbon of the substrate to the enzyme, PQQ-like, co-factor. The reduced catalytically inactive form can then be reoxidised to the catalytically active form by binding oxygen to which the two electrons are passed to form and release H_2O_2. The precise role of copper is unclear but it is essential for the first part of the reaction. A greater understanding will become apparant with unequivecal identification of the co-factor of lysyl oxidase.

1.8.7 Inhibition by β-aminopropionitrile (βAPN)

In 1933 it was found that connective tissue disorders could be induced by feeding rats on sweet peas (*Lathyrus odoratus* ; Geiger *et al.*, 1933). The compound responsible for this effect was found to be βAPN (Schilling and Strong, 1954). Lysyl oxidase was found to be inhibited by βAPN which consequently inhibited cross-linking in collagen and elastin (Siegel *et al.*, 1970a). Fifty percent inhibition occurred at concentrations of 3-5 μM βAPN (Narayanan *et al.*, 1972). βAPN initially inhibits lysyl oxidase competitively but eventually it binds covalently to the active site and inhibition becomes irreversible (Tang *et al.*, 1983).

1.8.8 Precursor form

Recently a precursor of lysyl oxidase (M_r = 40-48K) has been found, in fibrotic rat liver (Wagasaki and Ooshima, 1990a), in rat skin and uterine tissue (Romero-Chapman *et al.*, 1991) and rat aorta smooth muscle cells (Trackman *et al.*, 1992). The cDNA sequences of the rat aorta lysyl oxidase precursor (Trackman *et al.*, 1990; Trackman *et al.*,1991), the human lysyl oxidase precursor (Hamalainen *et al.*, 1991), and the chick aorta lysyl oxidase precursor (Wu *et al.*, 1992) have also been described.

The precursor from rat aorta smooth muscle cells (M_r = 50K) is N-glycosylated (Trackman *et al.*, 1992; Section 1.8.1), though the active enzyme is not glycosylated. N-glycosylation therefore occurs in the propeptide region, although a N-glycosylation recognition sequence has been identified in the 32K enzyme region. A 47K non-glycosylated enzyme precursor has also been identified in cell cultures (Trackman *et al.*, 1991). Thus the glycosylated structure on the 50K precursor is believed to have M_r = 3K. The 50K protein is soluble and believed to be secreted into the extracellular matrix where it is cleaved to give the 32K lysyl oxidase (Trackman *et al.*, 1992). The precise site of cleavage is controversial (Chapter 3).

1.8.9 Hormonal control and other mechanisms

The activity of lysyl oxidase is also under hormonal control. For example, in hypophysectomised rats, (i.e. rats in which the hypophysial blood vessels, which link the hypothalmus to the pituitary gland, are removed), lysyl oxidase activity and cross-linking *in vivo* were reduced, which indicates the effect of pituitary hormones on lysyl oxidase (Shoshan and Finkelstein, 1976). Lysyl oxidase activity in rat skin was also found to be 91% inhibited by injection of a synthetic glucocorticoid (Benson and LuValle, 1981). The two effects may be related as corticosteroids inhibit secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland.

As a further example of hormonal control, synthetic oestrogen was found to increase lysyl oxidase activity in skin and bone when supplied to

ovariectomized rats. Oestrogen also increases the activity of mouse cervical lysyl oxidase (Ozasa *et al.*, 1986). Testosterone increases lysyl oxidase activity in skin, but not bone, in ovariectomized rats (Sanda *et al.*, 1978), and testosterone also increases activity in cultured aortic smooth muscle cells (Bronson *et al.*, 1987). Hormone-receptor complexes may regulate transcription of lysyl oxidase DNA (Vannice *et al.*, 1984).

Lysyl oxidase activity may also be controlled by interaction with other matrix components. For example, a lysyl oxidase enhancer protein has been isolated from porcine skin and shown to enhance lysyl oxidase activity up to 3 fold *in vitro*. The identity of this protein is unknown (E. Forbes, personal communication).

1.8.10 Nutritional effects

Starvation of rats for 15 hours decreased lung lysyl oxidase activity by approximately 25% compared to controls (Madia *et al.*, 1979), and normal lysyl oxidase activity was restored three hours after re-feeding. Thus lysyl oxidase activity may be modulated by a poor diet. Copper deficiency is the likely cause of this effect as shown by 95% decrease in lysyl oxidase activity observed when chicks were fed on a copper-deficient diet (Harris *et al.*, 1982). When $CuSO_4$ was subsequently injected into these animals 50-100% lysyl oxidase activity was restored after 6-20h. It is thought that copper may have a regulatory role in the biosynthesis of lysyl oxidase in addition to being a co-factor (Harris *et al.*, 1982).

Ascorbate levels may also regulate lysyl oxidase activity (Faris *et al.*, 1984). When supplied in low concentrations (0.5 μg/ml) to ascorbate deficient cultures of rabbit smooth muscle cells, lysyl oxidase activity and soluble elastin levels remained constant but collagen levels increased. At higher levels of ascorbate, both lysyl oxidase activity and soluble elastin synthesis decreased, whilst collagen synthesis increased further.

1.8.11 Pathology

1.8.11.1 Introduction

There are several inherited diseases which lack or have reduced levels of lysyl oxidase, and these result in symptoms that resemble induced lathyrism. However, in many cases, it is likely that the deficiency of lysyl oxidase is due to a disruption of copper metabolism. In contrast, there are a number of diseases characterised by an excess of lysyl oxidase. Such conditions lead to an excessive amount of collagen and elastin in tissues (fibrosis). A number of diseases which were previously attributed to lysyl oxidase deficiency, e.g. Marfan's syndrome and osteogenesis imperfecta, have recently been found to be due to other causes. Marfan's syndrome is a dominantly inherited connective tissue disorder, characterised by musculoskeletal and cardiovascular abnormalities and lens dislocation. Most cases of Marfan's syndrome are caused by mutations in the fibrillin gene on chromosome 15 (FBN 1) which leads to low concentrations of fibrillin in cells and tissues (Dietz *et al.,* 1991). Several forms of osteogenesis imperfecta, characterised by, brittle bones, hearing loss and blue sclerae have been shown to be caused by mutations in type I collagen (Byers, 1990).

1.8.11.2 Diseases due to deficiency of lysyl oxidase

At least five mutations in the mottled locus on the murine X-chromosome have been described (Royce and Steinmann, 1990). Some of the phenotypes are lethal. The blotchy male variant exhibits bone deformities, extensible skin, aortic aneurisms (swelling and distension of the aortic wall) and defects in lung elastin. The viable brindled variant shows a decrease in desmosine concentration in aortic elastin. In both variants, lysyl oxidase activity is reduced, by 50% and 67% respectively, and this is due to a disruption of copper metabolism (Rowe *et al.*, 1974). These symptoms resemble Menke's syndrome in humans.

Menke's syndrome is a lethal X-linked, recessively inherited disorder (Royce and Steinmann, 1990). It has a frequency of 1 in 50,000 to 100,000

births. This disorder is characterised by growth retardation, severe cerebral degeneration, kinky hair, hypothermia and generalised arterial disease. The lysyl oxidase activity in Menke's fibroblast cultures was reduced by more than 50% compared to controls. In a severe case of the disease skin and aorta lysyl oxidase was reduced by 86-94% of control levels. Again, the enzyme deficiency appears to be secondary to disturbances in copper metabolism.

Ehlers-Danlos syndrome is a group of inherited connective tissue disorders characterised by joint hypermobility, hyperextensibility, thinness and fragility of skin. The syndrome has been divided into 10 subtypes according to clinical, genetic and biochemical criteria. Ehlers-Danlos syndrome subtype IX shows similar symptoms to Menke's syndrome, but the patient's hair is coarse, not kinky, and there are no neurological abnormalities. Copper content is low in skin and hair and there are reduced levels of lysyl oxidase in the media of the cultured fibroblasts form affected individuals. Ehlers-Danlos syndrome subtype V was thought to be caused by a defect in the lysyl oxidase gene (Siegal, 1979), though this is now thought to be unlikely (Kagan, 1986).

Cutis laxa is a rare, heterogeneous disorder of elastic tissues affecting the skin, cardiovascular system and lungs (Uitto *et al.,* 1982). It may be inherited as an autosomal dominant, autosomal recessive or a X-linked recessive disease, or it may occur as an acquired form. The molecular basis of the disease remains elusive. In all cases examined so far, light and electron microscopy have shown that dermal elastin content is severely reduced, which suggests that the abnormality might lie in elastin metabolism. Biochemical and molecular analysis of the fibroblasts have confirmed defects in elastin synthesis or gene expression in some but not all cases examined. However, in some patients alteration of the collagen component, such as fusion of fibrils, has been described. Deficiency of lysyl oxidase has been observed in cultured fibroblasts from two patients with X-linked cutis laxa (Byers *et al.*, 1980). The reduction in lysyl oxidase activity may be a secondary effect of deficient copper metabolism.

Some of the above deficiencies in lysyl oxidase activity can be at least be partially reversed. For example, subcutaneous injections of copper to the brindled aneurism prone mouse was found to increase lysyl oxidase activity (Royce *et al*, 1982). Furthermore, administration of propanolol was found to

stimulate lysyl oxidase activity in aneurism prone turkeys (Boucek *et al.*, 1983).

1.8.11.3 Diseases due to an excess of lysyl oxidase

Excess lysyl oxidase activity is associated with a number of cardiovascular diseases. For example, early lesions in atherosclerotic arterial walls are associated with greater deposition of collagen and elastin, lysyl oxidase levels increasing 2.5 times in a rabbit model of atheroscleosis (Kagan *et al.*, 1981). Collagen biosynthesis and deposition on vascular walls also increases in hypertensive rats (Ooshima *et al.*, 1974), and treatment with βAPN restores collagen biosynthesis to normal levels (Sheridan *et al.*, 1979). It is possible, therefore, that anti-fibrotic drugs, through their effect on lysyl oxidase, may be useful in the treatment of hypertension. Myocardial lysyl oxidase activity also increases in experimental rabbits after induction of myocardial infarction (Lerman *et al.*, 1983).

Lysyl oxidase is also important in lung disease. For example, lung fibrosis can be induced by exposure to bleomycin sulphate (Counts *et al.*,1981), and this is associated with a six-fold increase in lysyl oxidase activity. Lung fibrosis can also be induced by inhalation of cadmium vapours, where lysyl oxidase activity increases 14-fold (Chichester *et al.*, 1981). The cadmium-induced lysyl oxidase has been isolated and resolved into four peaks of activity when separated by anion exchange (Almassian *et al.*, 1991). When cadmium chloride is fed to chicks, bone lysyl oxidase activity decreases. This is thought to be due to cadmium being incorporated into lysyl oxidase in preference to copper (Harris, 1986).

Increased collagen synthesis and deposition is found in human liver from alcoholic patients (Chen and Leevy, 1975). Liver fibrosis can be induced by repeated injections of carbon tetrachloride into rats. It was found that hepatic lysyl oxidase activity increased 30 times in such circumstances (Siegel *et al.*, 1978). Assay of serum lysyl oxidase activity has been found to be a sensitive indicator of liver fibrosis. Activity increased 1.6 fold in chronic persistent hepatitis, 4.4 fold in chronic active hepatitis and 11.8 fold in cirrhosis, when compared to controls (Murawaki *et al.*, 1991).

Drug induced diabetes in rats leads to increased lung lysyl oxidase activity, collagen biosynthesis and deposition (Madia *et al.*, 1979). The activity of other enzymes involved in collagen biosynthesis are also increased (Kagan, 1986). It has been found in the skin collagen from patients with insulin-dependent diabetes mellitus (IDDM), that the amounts of the lysyl oxidase dependent difunctional cross-link dihydroxylysinonorleucine (DHLNL) and the trifunctional maturation product hydroxypyridinium (OHP), both increase, while all other cross-links remain normal. The skin also shows an increase in non-enzymic glycosylation of lysine and hydroxylysine which results in the formation of hexosyl-lysine and hexosyl-hydroxylysine. Some of these early products are believed to form Schiff bases and then undergo an amido-rearangement to form ketoamines (Buckingham and Reiser, 1990). The non-enzymic glycosylation of collagen may affect formation of specific lysyl oxidase dependent cross-links and this may cause long term skin and cardiovascular complications for diabetic patients.

The administration of βAPN as a means of controlling fibrosis has been studied in various tissues (Kagan, 1986). As βAPN is a powerful inhibitor of lysyl oxidase, care must be taken not to over-prescribe and thereby lead to loss of tensile strength.

1.9 Tyrosine Rich Acidic Matrix Protein (TRAMP)

Tyrosine Rich Acidic Matrix Protein (TRAMP) is a new protein of the extracellular matrix (M_r = 24K) that has been found to co-purify with lysyl oxidase from porcine skin (Chapter 3; Cronshaw *et al.*, 1993). The purification of lysyl oxidase involves an initial DEAE ion exchange chromatography step followed by an unususal chromatographic procedure in which the enzyme binds selectively to Sephacryl S-400 an a low ionic strength neutral buffer. When eluted from the Sephacryl column, lysyl oxidase (M_r = 32K) appears with a 24K contaminant. Such a contaminant had previously been reported in the lysyl oxidase literature (Sullivan and Kagan, 1982; Kuivaniemi *et al.*, 1984; Burbelo *et al.*, 1986) and it was thought to be a degradation product of the enzyme. The work discussed in this thesis shows that the 24K

contaminant in porcine skin lysyl oxidase is a distinct protein, which, because of its high tyrosine content and relatively low isoelectric point, has been called TRAMP (Tyrosine Rich Acidic Matrix Protein). Analysis of the amino acid sequence of TRAMP (Chapter 3) shows that it is almost identical to a bovine 22K extracellular matrix protein, which co-purifies with dermatan sulphate proteoglycans (Choi *et al.*, 1989; Neame *et al.*, 1989).

The function of TRAMP is not well understood. It appears to have a widespread tissue distribution (present in extracts of heart, lung, kidney, spleen, skin, brain, skeletal muscle and liver; E. Forbes, unpublished observations). A possible role in cell adhesion has been suggested for the equivalent bovine 22K protein (Lewandowska *et al.*, 1991). TRAMP does not affect lysyl oxidase activity on an elastin substrate, nor does it affect the activity of added lysyl oxidase (Cronshaw *et al.*, 1993). TRAMP may be involved in the assembly of collagen fibrils, as it has been found to accelerate this process *in vitro* (MacBeath *et al.*, 1993). Additional properties of TRAMP are discussed in Chapters 3 and 4.

Chapter 2 Materials and methods

2.1 Lysyl oxidase assay

The assay for lysyl oxidase (Shackleton and Hulmes, 1990b) involves incubation of enzyme with an elastin substrate that has been labelled biosynthetically with [4,5-^{3}H]lysine. As a result of the reaction, tritium exchanges with water and the ^{3}HHO formed is separated by ultrafiltration using Millipore Ultrafree-MC microconcentrators. Elastin is preferred as a substrate as there are approximately 25 residues per molecule available for lysyl oxidase activity compared to only a few residues per molecule in collagen. Elastin is also more accessible as a substrate (collagen must be in fibrillar form), it is easier to produce and assay results are comparable to other assays. The disadvantage of using elastin is that due to its particulate nature, both the molar concentration and the extent of cross-linking, are unknown.

For each assay, a 100 µl aliquot [^{3}H]elastin substrate (containing 3 x 10^{5} d.p.m.) suspended in assay buffer (0.1M $Na_2B_4O_7$, 0.15M NaCl, pH 8.0), was placed in a 1.5 ml microcentrifuge tube. To this, 700 µl assay buffer and 100 µl enzyme were added. The reaction mixture was vortexed and then incubated at 37°C for 16h. The reaction was then stopped by the addition of 100 µl 50% (w/v) trichloroacetic acid to give a final concentration of 5% (w/v). The bulk of the elastin substrate was then pelleted by centrifugation (IEC Centra-M-Microcentrifuge) at 15,600 g at 2°C for 5 min. After this, 420 µl of the supernatant was transferred to a Millipore Ultrafree-MC cellulose low binding 10,000 NMWL microconcentrator, which was then centrifuged at 4,200 g at 2°C for 1.5h in a Beckman JA-18.1, 45° fixed angle rotor. During centrifugation, low molecular weight material passed through the ultrafiltration membrane and collected in the lower chamber of the microconcentrator. To measure the tritium release, 300 µl of the ultrafiltrate was mixed with 2.7 ml Cocktail "T" scintillation fluid (BDH) in a plastic scintillation vial insert and then counted (for 10 min) in a Packard Model 1900CA Tri-carb liquid scintillation counter. Control assays contained 0.2 mM β–aminopropionitrile (βAPN), a specific inhibitor of lysyl oxidase.

Partial inhibition of lysyl oxidase activity in the presence of urea (0.33M, 0.67M) was corrected by multiplying observed c.p.m. (above background) by the appropriate correction factors (1.40 and 2.33 respectively)

based on the linear inhibition of lysyl oxidase activity by urea concentrations up to 1M (Shackleton and Hulmes, 1990b).

2.2 Protein assay

The protein concentrations of each sample were assayed by one of two methods, depending on the approximate protein concentration. If adequate protein was present a Bradford assay was used (Bradford, 1976). A standard was established using 10-100 μg/ml bovine serum albumin (BSA). To each aliquot (100 μl of BSA solution or sample) 1 ml Bradford Reagent (0.01% (w/v) Coomassie Brilliant Blue G-250; 4.7% (w/v) ethanol; 8.5% (w/v) phosphoric acid) was added, mixed and allowed to stand at room temperature for 30 min. After this time the absorbance at 595 nm was measured using an LKB Model 4050 Ultrospec II uv/visible specrophotometer. If protein was present in low concentrations a micro bicinchoninic acid (BCA; Pierce) protein assay was used (Smith *et al.*, 1985). A standard curve was established using 1-20 μg/ml bovine serum albumin (BSA). To each aliquot (50 μl BSA solution or sample) 1 ml Micro BCA Reagent was added, mixed and allowed to incubate at 60°C for 60 min. After this time the absorbance at 562 nm was measured. For plots of each assay absorbance versus known protein concentration were fitted by linear regression analysis. Where necessary the starting protein solutions were diluted to bring them within the range of the standards.

2.3 Preparation of lysyl oxidase and TRAMP

2.3.1 Materials

Stillborn piglets were obtained from the Animal Breeding Research Organisation, Dryden-Montmarle Field Laboratory, Roslin, Midlothian, U.K. The skins were removed within 24h of death and stored at -20°C for up to one week before use. CM-Sepharose Fast Flow, DEAE-Sepharose Fast Flow,

Sephadex G-25 (medium grade), Sephacryl S-400, Mono Q HR5/5 and ProRPC HR5/5 columns were purchased from Pharmacia Biotechnology Ltd., Milton Keynes, Bucks., U. K. β–aminopropionitrile, formate salt (βAPN) was from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. All other chemicals (analytical grade) were from B.D.H., Merck Ltd., Poole, Dorset, U.K.

2.3.2 Deionisation of urea

All buffered urea solutions were prepared from fresh stock solutions of 8M urea which had been deionised at 4°C with BioRad AG 501-X8 mixed bed resin immediately before use. The beads were removed from urea by filtration through Whatman No.113v filter paper.

2.3.3 Preparation of dialysis tubing

Approximately 2 m lengths of Visking cellulose tubing (BDH), were boiled in 0.1% $NaHCO_3$ for 30 min, and washed in water before use. The glassware used to contain the dialysis tubing was soaked in concentrated HCl before use and then extensively washed with purified water (Section 2.8.10).

2.3.4 Extraction of lysyl oxidase and TRAMP

Piglet skin lysyl oxidase was prepared using a procedure similar to that described by Shackleton and Hulmes (1990a) but with the following modifications to improve separation, increase capacity, and reduce the time required. Typically, skins from ten stillborn piglets were used (approximately 1 kg wet weight). All extraction and purification steps were carried out between 0 and 4°C. The skins were fed through a fine mincer before homogenisation in phosphate buffered saline (PBS; 0.09M Na_2HPO_4/0.01M NaH_2PO_4/0.15M NaCl, pH 7.8), at 0.5 ml/g tissue, in a Waring Commercial Blender for 30s. The volume of homogenate and buffer was sufficient to fill six 250 ml centrifuge tubes. The homogenate was then centrifuged in a Beckman J2-21 centrifuge, using a JA-14 fixed-angle rotor, at 12,400 g for 20 min, and the supernatant

was discarded. This extraction was repeated twice. The pellet was then washed three times in phosphate buffer (PB; 9mM Na_2HPO_4/1mM NaH_2PO_4, pH 7.8) at 0.5 ml/g, with homogenisation and centrifugation as above. The pellet was then extracted four times in 6M PBU (PB containing 6M urea, pH 7.8) at 0.5 ml/g of original material, for 8-18h intervals, and centrifuged as above. The four supernatants were pooled after recording their volumes. This supernatant extract (E) was first filtered through a Whatman No.113v folded filter paper (32 cm diameter) followed by filtration through a Whatman 3MM filter paper (15 cm diameter), using a Buchner funnel and flask, before the first chromatography step (Fig. 3.1).

2.3.5 Purification of lysyl oxidase and TRAMP from the extract by large scale chromatography

The filtered extract (E) was diluted with PB to 2M PBU and loaded onto a CM-Sepharose Fast Flow column (5 cm x 25 cm; volume = 500 ml), previously equilibrated with 2M PBU, at a flow rate of 30 ml/min. The buffer was applied to the column using a Watson-Marlow model 502S peristaltic pump. This procedure bound and hence removed unwanted proteins. The flow-through solution was then immediately applied to a DEAE-Sepharose Fast Flow column (5 cm x 25 cm; volume = 500 ml), previously equilibrated with 2M PBU, at a flow rate of 30 ml/min. The DEAE-Sepharose column was then washed with two column volumes of 2M PBU, followed by two column volumes of PB, and then most of the bound proteins (but not lysyl oxidase or TRAMP) were eluted with PB containing 0.3M NaCl. Fractions were collected in 50 ml tubes with the aid of a Central Ignition Company fraction collector. Hereafter 1 ml aliquots from fractions eluting from this column were assayed for absorbance at 280 nm using an LKB Ultrospec II Model 4050 uv/visible spectrophotometer. To release bound proteins containing lysyl oxidase activity but relatively uncontaminated with TRAMP, the column was eluted, at 30 ml/min, with 3M PBU containing 0.3M NaCl. A subsequent elution with 6M PBU containing 0.5M NaCl released predominantly TRAMP. Lysyl oxidase and TRAMP were further purified separately, as described below.

For further purification of lysyl oxidase, the 3M PBU/0.3M NaCl eluate

from the DEAE-Sepharose column was passed through a Sephadex G-25 medium grade gel filtration column (5 cm x 100 cm, volume = 2 litre) previously equilibrated with PB, at a flow rate of 40 ml/min to remove the urea and salt. Afterwards 1 ml aliquots of the eluted fractions were measured for absorbance at 280 nm. The protein peak was pooled and loaded onto a Sephacryl S-400 column (5 cm x 20 cm; volume = 400 ml), previously equilibrated with PB, at a flow rate of 15 ml/min. (Sephacryl S-400 was used instead of Sephacryl S-200 (Shackleton and Hulmes, 1990a), with no adverse effect on recovery of enzyme activity). The column was washed with PB until a steady reading of absorbance at 280 nm was obtained. Finally, bound proteins were eluted with 1.5M PBU, followed by 6M PBU. Lysyl oxidase appeared in the 6M PBU eluate.

For further purification of TRAMP, the 6M PBU/0.5M NaCl eluate from the DEAE-Sepharose column was used. Urea and NaCl were removed by gel filtration, using the Sephadex G-25 column, and the protein solution in PB was loaded onto the S-400 column as above. The column was washed with PB, then bound proteins were eluted with 1.5M PBU, followed by 6M PBU. TRAMP appeared in the 1.5M PBU eluate and a mixture of lysyl oxidase and TRAMP was found in the 6M PBU eluate. After use all columns were stored in 20% ethanol.

(Note: The mechanism of the interaction between Sephacryl and lysyl oxidase or TRAMP is not clear. It may be an electrostactic interaction due to the relatively acidic nature of both proteins, or it may be due to the formation of large molecular weight aggregates of these proteins in the absence of urea, or a combination of both these effects).

2.4 Fast Protein Liquid Chromatography (FPLC) and High Pressure Liquid Chromatography (HPLC)

2.4.1 Introduction

The techniques of FPLC and HPLC have been used extensivly to purify mixtures of proteins and peptides. It was important to ensure that the columns

used were cleaned before and after use. Prior to running any samples the columns were equilibrated with the appropriate buffers. After this a blank run followed by a standard run were made. Only if these runs were satisfactory would a sample run be made. All digest procedures were carried out in the absence of protein in parallel with normal digests. These digest blanks were run on reverse phase columns prior to sample digests, which enabled clearer interpretation of chromatograms. Individual components of a digest mixture, e.g. clostripain, could be run separately to locate their retention positions and thus ensure that they did not co-elute with peptides of interest.

Purified water and Applied Biosystems Incorporated (ABI) chemicals were used to prepare solvents for the Applied Biosystems 130A Microbore Separation System (Section 2.8.10). The quality of reagents required for FPLC were not so stringent, so buffers were made up in double distilled water. Before use these buffers were filtered through a cellulose acetate 0.22 μm Millipore filter. After use anion exchange and chromatofocussing columns were stored in 24% ethanol and reverse phase columns stored in methanol.

2.4.2 Separation of different variants of lysyl oxidase and TRAMP by anion exchange chromatography

Partially purified lysyl oxidase was further purified by loading onto a Pharmacia Mono Q HR5/5 anion exchange column (10 μm particle size; 5 mm x 50 mm) previously equilibrated with 6M PBU. When a linear gradient from 0 to 1M NaCl in 6M PBU was applied, at a flow rate of 2 ml/min, four forms of lysyl oxidase were isolated (Chapter 3). When TRAMP was treated in the same way, five forms were isolated (Chapter 3).

2.4.3 Removal of urea from lysyl oxidase and TRAMP variants by reverse phase chromatography

Two columns were employed for this procedure according to the amount of protein present. For preparative amounts of protein a Pharmacia ProRPC HR5/2 (C1/C8) reverse phase column (5 μm particle size; 5 mm x 20 mm) was used at a flow rate of 2ml/min. For analytical amounts the column of

choice was an Applied Biosystems 130A Separation System containing an Aquapore RP-300 C8 cartridge (7 μm particle size; 2.1 mm x 30 mm) at a flow rate of 200 μl/min. In each case the columns were previously equilibrated with solvent A (aqueous, 0.1% (v/v) trifluoroacetic acid (TFA)), and eluted with a linear gradient from 0 to 70% solvent B (100% acetonitrile containing 0.08% (v/v) TFA).

2.4.4 Separation of lysyl oxidase and TRAMP peptides by reverse phase chromatography

For separations of lysyl oxidase and TRAMP peptides, the above procedure (Section 2.4.3) with the Aquapore RP-300 C8 cartridge was satisfactory. For improved resolution, however, a larger RP-300 C8 column (7 μm particle size; 1mm x 250 mm) was used at a flow rate of 100 μl/min, with the same loading and similar elution conditions.

The standard protein mixture used to check resolution of each of these columns contained the following proteins: insulin, cytochrome c, lactalbumin, carbonic anhydrase, and ovalbumin (ABI).

2.4.5 Measurement of the elution pH of lysyl oxidase and TRAMP by chromatofocussing chromatography

For chromatofocussing, samples (1ml) in 1M urea were loaded on to a Pharmacia Mono P HR5/20 chromatofocussing column (10 μm particle size; 5 mm x 200 mm) previously equilibrated with 0.025M Bis-Tris, pH 4.70 (adjusted with HCl). A pH gradient was achieved by applying Pharmacia Polybuffer 74, diluted 1:10 with water, pH 3.15, (adjusted with HCl), from a 50 ml "superloop". A flow rate of 1 ml/min was used, and absorbance was monitored continuously at 280 nm. The elution pH of TRAMP was found similarly but the pH values for the two buffers used were pH 3.65 and pH 2.60, respectively. The pH of each 0.5 ml fraction was measured. The urea passed straight through the column leaving the protein to be eluted at its elution pH which is dependent on its isoelectric point (pI; Fagerstam *et al.,* 1983).

After each run 1 ml 2M NaCl was injected onto the column to remove

any bound substances. The column was then re-equilibrated with start buffer until the effluent had reached the correct pH.

Initially, in this study, a wide pH interval of 7-4 was chosen. It was found, however, that TRAMP was still bound to the the column at pH 4.00 and only eluted when the pH was dropped to 2.20. Gradually a narrow pH interval was found in which the different forms of TRAMP were resolved. Care was taken at all times to avoid reaching pH 2.00 whereby the stability of the column would have been affected.

Before any samples were run the column performance was checked by loading 25 µl 0.2 mg/ml triglycine (Sigma), using a mobile phase of 0.005M NH_4Cl, pH 9.90, at a flow rate of 0.5 ml/min, monitoring at 214 nm absorbance.

2.5 Electrophoresis

2.5.1 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE (modified from the method of Laemmli, 1970) was carried out in polyacrylamide gels, where %T = total acrylamide + bis-acrylamide (g/100 ml) and %C = cross-linker (bis-acrylamide, g/100ml).

Two types of gel electrophoresis apparatus were used. The first type was a Hoefer SE 280 Tall Mighty Small 12 cm vertical slab mini gel system. The second type was a LKB large format 18 cm x 18 cm gel unit. The composition of the gels in each case remained the same but the volumes used were greater for the large format gels.

2.5.2 Gel composition

2.5.2.1 12% Separating gel

Separating gel buffer was prepared by dissolving 36.34 g Tris base (BioRad) and 0.8 g SDS in distilled water adjusting pH to 8.8 (with HCl) and

making up the volume to 200 ml. The solution was then filtered through a 0.2 µm Millipore filter, and was stored at 4°C. The acrylamide stock was prepared by dissolving 30 g acrylamide (BioRad) and 0.8 g bis-acrylamide (BioRad) and making the volume to 100 ml. This solution was filtered through a 0.22 µm filter and stored at 4°C in a dark bottle.

The 12% separating gel was prepared by mixing 1.50 ml separating gel buffer, 2.40 ml acrylamide: bis-acrylamide stock, 2.07 ml distilled water, 3 µl TEMED (BioRad) and 100 µl 10% (w/v) ammonium persulphate. (The ammonium persulphate was made up fresh and was added last.) The volumes were scaled up seven times for the large format gels.

The percentage acrylamide composition of the separating gel was varied from 10 to15% (%T = 10.027; %C= 0.027 to %T = 15.041; %C = 0.041) with the higher percentage acrylamide used for the separation of low molecular weight proteins.

2.5.2.2 Stacking gel

The stacking gel buffer was prepared by dissolving 12.11g Tris base and 0.8g SDS in water and adjusting the pH to 6.8 with HCl, and making up the volume to 100 ml. The solution was filtered through a 0.22 µm Millipore filter and stored at 4°C.

The stacking gel was prepared by mixing 0.75 ml stacking gel buffer, 0.5 ml acrylamide: bis-acrylamide stock, 1.75 ml distilled water, 2 µl TEMED, and 21 µl 10% (w/v) ammomium persulphate. The volumes were scaled up seven times for the large format gels.

2.5.3 Sample preparation

Sample buffer was prepared by mixing 1.0 ml glycerol (BRL), 0.5 ml 2-mercaptoethanol (Sigma), 0.3 g SDS, and 1.25 ml stacking gel buffer and making the volume up to 10.0 ml with distilled water. One volume of each protein sample was mixed with one volume of sample buffer. An aliquot of 2µl 5% bromophenol blue (Bio-Rad; dissolved in 50% methanol) was added to each sample, and the mixture boiled at 100°C for 5min.

Aliquots of 10µl of protein sample containing 2-20 µg of protein were loaded into each of the mini gel wells. Aliquots of 60 µl of each sample containing 10-100 µg protein were loaded into each of the large format gel wells.

2.5.4 Assembly of gel cassette and pouring of gels

Two gel plates were washed with methanol, dried then clamped together separated by 0.75 mm or 1.5 mm thick teflon spacers. The bottom edge of the gel was sealed by placing it upon a strip of molton 1% agar (Sigma), poured out onto a horizontal glass plate. Once the agar had set, 12% separating gel solution was carefully poured between the glass plates and overlaid with a water saturated butan-2-ol solution. Upon polymerising, the butan-2-ol/water solution was washed away with several washes of distilled water. The stacking gel solution was carefully poured into the remaining space between the glass plates and a teflon comb inserted. After polymerisation the teflon comb was removed and the cassette mounted onto the lower gel tank reservoir section. The upper and lower reservoirs were filled with electrophoresis buffer. This buffer comprised 3.0 g Tris, 14.4 g glycine, and 1.0 g SDS per litre of distilled water. Treated proteins were loaded into each of the wells. A solution of SDS-PAGE low molecular weight markers, containing approximately 4 µg of each protein, were loaded into the first and last of the sample wells each time. The large format gels used an additional spacer, placed horizontally, instead of using agar.

The lid was placed on the gel tank and the mini gels were run with a constant current of 25 mA for approximately 1.5h. In contrast, the large format gels were run at a constant current of 40 mA for approximately 3h. When the bromophenol blue dye-front had reached the base of the gel, the power supply was switched off, the gel cassette was removed, taken apart and the gel was placed in gel fixing solution (10% (v/v) acetic acid, 45% (v/v) methanol). After fixing (15 min for mini-gels, 1h for large format gels), gels were stained with Coomassie Blue, silver stain or Alcian Blue.

2.5.5 Coomassie Brilliant Blue staining

The gels were stained with Coomasie Blue (0.02% (w/v) Coomassie Brilliant Blue R-250 (BDH) in 7.0% (v/v) glacial acetic acid, 50% (v/v) methanol) for 10 min in the case of the mini gels and for 2 hours in the case of large gels. Each type of gel was destained overnight in several changes of gel destaining solution (10% (v/v) glacial acetic acid, 10% (v/v) methanol; Miller and Rhodes, 1982).

2.5.6 Silver staining

Some SDS-PAGE gels were silver stained (Wray *et al.*,1981). Silver stain is at least 50 times more sensitive than Coomassie Blue stain allowing 10 ng of protein to be detected. Gels to be stained were fixed and soaked in three changes of 50% (v/v) methanol over a period of 3 hours to remove glycine remaining from in the electrophoresis buffer. During this process gels shrank to about one third of their original size and so were re-hydrated in two changes of distilled water.

All glass containers were throughly washed with methanol prior to use and were solely used for silver staining. Silver staining solution was prepared by adding a solution of 0.4 g $AgNO_3$ (BDH) in 2 ml distilled water dropwise, with continuous stirring, to a mixture of 21 ml 0.36% (w/v) NaOH and 1.4 ml 14.8M NH_4OH, and then making up the volume to 100 ml with distilled water. This solution was used within 5 min of preparation. The gel was stained at room temperature in silver stain solution for 15 min with constant agitation. The stain solution was then discarded and the gel washed for 5 min each in four changes of distilled water. The gel was developed in a freshly prepared solution containing 2.5 ml of 1% (w/v) citric acid and 250 µl 38% (v/v) formaldehyde (BDH), made up to 500 ml with distilled water, for 10-15 min with constant agitation, until brown stained bands were visible. The development was stopped before the background became too intensly stained by replacing this solution with a stop solution of 10% glacial acetic acid in 50% (v/v) methanol. If the gel had been overstained it was destained for 1 to 5 min. Destain solution was prepared as follows: 3.7 g NaCl and 3.7 g

cupric sulphate were dissolved in 85 ml distilled water. A few drops of 14.8M NH_4OH were added until a deep blue colour was observed and the solution was made up to 100 ml with distilled water. A second solution of 43.6 g sodium thiosulphate (BDH) in 100 ml distilled water was then prepared and mixed with an equal volume of the first solution to make destain. The reaction was stopped with 10% acetic acid and the gel stored in water .

2.5.7 Alcian Blue staining

Alcian Blue stains sulphated polyanions. After electrophoresis gels were fixed in 50% (v/v) methanol, 7% (v/v) acetic acid for 1h. The gels were then washed in distilled water for 1h. This fixing step followed by washing was repeated once in order to remove SDS from the gels. Stain precipitation can occur in the staining solution or on the gel surface if SDS is not first removed. The gels were stained overnight in 0.2% (v/v) Alcian Blue 8GX, 3% (v/v) acetic acid, 0.05M $MgCl_2$. Gels were destained in several changes of 3% (v/v) acetic acid, 0.05M $MgCl_2$ until the background became clear. The $MgCl_2$ was added to the stain and destain solutions to eliminate background staining according to the critical electrolyte concentration (Wall and Gyi, 1988).

2.5.8 Calibration of SDS-polyacrylamide gels

The molecular weights of the proteins separated on SDS-polyacrylamide gels were determined by comparing their mobilities to those of a set of low molecular weight markers (Pharmacia). The markers were reconstituted in 200 µl of sample buffer and incubated at 100°C for 5 min (Section 2.5.3). They were stored as 50 µl aliquots at -20°C.

The marker kit was composed of the following proteins: phosphorylase b (M_r = 94.0K), albumin (M_r = 67.0K), ovalbumin (M_r = 45.0K), carbonic anhydrase (M_r = 30.0K), trypsin inhibitor (M_r = 20.1K), and α–lactalbumin (M_r = 14.4K).

2.5.9 Gel scanning

After gels had been stained with Coomassie Brilliant Blue, the migration distance of the bands and the relative amounts of protein were quantifed by scanning densitometry with a Joyce Loebl Chromoscan 3 at 530 nm using an aperture width of 0.1 mm.

2.5.10 High voltage paper electrophoresis (HVPE)

High voltage paper electrophoresis (HVPE) was used to attempt to identify suphotyrosine in alkaline hydrolysates of TRAMP (Section 3.3.8). Aliquots (5 µl) of 1mg/ml standards and samples were loaded for each run. Samples were applied to a Whatman 3MM filter paper (57 cm x 46 cm) and dried. A 5 µl aliquot of methyl green was added to one side of the sample as a positively charged marker for runs in pH 2.1 and pH 3.5 buffers so that progress of the separation could be monitored during the run. Orange G was used as a positively charged marker in the pH 6.5 buffer system. The Whatman paper was wetted with a suitable buffer (see below), placed in a Michl tank apparatus containing coolant, and a potential gradient of 3kV was applied across the paper for 40 min (by M. Daniel, Institute of Cellular and Molecular Biology, University of Edinburgh). Charged molecules migrate at a rate approximately proportional to their charge to mass ratio (Ambler, 1963).

The buffers used had adequate buffering capacity and conductvity so that the presence of the sample did not affect the pH or the potential gradient. The buffers were completely volatile, so that no residue was left in the paper to interfere with subsequent operations. The buffer systems used were as follows:

(a) pH 2.1 (formic acid: acetic acid: water; 10: 35: 355; v/v) with white spirit used as coolant.

(b) pH 3.5 (pyridine: acetic acid: water; 1: 10: 89; v/v) with white spirit used as coolant. The buffer was diluted to half strength for wetting the paper.

(c) pH 6.5 (pyridine: acetic acid: water; 10: 1: 89; v/v) with toluene containing 8%(v/v) pyridine as coolant.

After electrophoresis, the paper was removed from the Michl tank and allowed to dry in an oven at 40°C. The paper was then dipped in 0.2% (w/v) ninhydrin in acetone to which 10 ml collidine: concentrated acetic acid (1: 2) was added. The collidine helped to produce a stronger colour reaction. The paper was then dried in air for 15 min before being placed in an oven at 100°C for 30s.

2.6 Amino acid analysis

2.6.1 Introduction

Pure proteins and peptides were subjected to amino acid analysis on an Applied Biosystems Model 420A Derivatiser with automatic hydrolysis with an on-line Model 120A phenylthiocyanate (PTC) analyser (Cronshaw *et al.*, 1993). Applied Biosystems chemicals and purified water were used throughout this automated analysis procedure (Section 2.8.10). The amino acid standard H (2.5 μmol/ml) was supplied by Pierce Chemicals.

2.6.2 Cleaning glassware

The successful operation of an ultrasensitive amino acid analyser demands great care in sample and reagent preparation. Dust and contact with fingers on glassware can introduce contamination and invalidate results.

Prior to use glassware was soaked overnight in a solution of Decon 90 and rinsed thoroughly in hot, cold and then distilled water. The glassware was then rinsed in 34.8 g/l Na_2EDTA solution followed by rinsing three washes with purified water (Section 2.8.10). The glassware was covered with cling film when stored. Magnetic stirring bars were cleaned similarly. Separate dedicated glassware was used to prepare each of the buffers. Microcentrifuge tubes were rinsed twice before use with two washes of purified water.

2.6.3 Sample preparation

Proteins and peptides which had been desalted by reverse phase chromatography were loaded onto scintered glass frits within a glass slide. Samples in the range of 200 pmol to 1 nmol of each amino acid were loaded. In some cases samples were hydrolysed manually and then loaded onto the analyser.

2.6.4 Manual hydrolysis

The samples were placed into the bottom of pyrolysed Pyrex test tubes (6 mm x 50 mm), in a total volume of 200µl 6M HCl (Pierce Sequanol grade). Then 500µl 6M HCl was placed at the bottom of a 25 ml borosilicate glass vial (27 mm x 70 mm; Pierce), and the sample tubes were placed in the vial and flushed with argon before being sealed with a black phenolic cap (size 24-240) lined with a "Tuf-Bond" teflon/silicone septum, teflon side down.

The vial was then placed in an oven at 110°C for 22h. After manual hydrolysis residual acid and condensation were removed by placing the sample tubes into a small vacuum desiccator containing NaOH pellets for 2h. The samples were then redissolved in 0.025% K_3EDTA prior to amino acid analysis (Dupont *et al.*, 1988).

2.6.5 Slide cleaning

New slides required preconditioning before use. This was achieved by loading 30 µl of 1 mg/ml K_3EDTA (ABI) solution onto each frit and running a precycle procedure. After this 5 µl (50 pmol) hydrolysis standard H was run on each frit, followed by 5 µl (500 pmol) test peptide (ABI), a peptide containing equal amounts of each standard amino acid. Once these frits were within the manufacturer's specification it was possible to proceed with analysis of unknown samples.

2.6.6 Automatic hydrolysis

The jaws of the hydrolysis head, top and bottom, closed around the three frits on a slide, and created a chamber around the sample which was deoxygenated by flushing with argon. The hydrolysis head allowed hydrolysis on the three frits simultaneously by delivering and heating 6M HCl to 200°C for 75 min. After hydrolysis the temperature of the head was decreased to 170°C. The lower internal vapour pressure facilitated the opening of the jaws. The slide was then rotated on the turntable to face the derivatiser head.

2.6.7 Automatic derivatisation

Once the sample had been hydrolysed, it was derivatised. Since free amino acids do not have a strong common spectral characteristic, they must be tagged with a reagent which will increase their detectability and make quantitation possible. The derivatisation process used in the amino acid analyser is the same as the first step of the Edman degradation used in the microsequencer. During precolumn derivatisation the free amino groups, either primary or secondary, are exposed to phenylisothiocyanate (PITC) under alkaline conditions, in the presence of diisopropylethylamine (DIEA), where they react quantitativly to form the phenylthiocarbamyl amino acid derivatives (PTC-AAs). The PTC-AA derivatives have a strong UV absorbance at 254 nm which allows picomolar detection of all amino acids. The derivatiser head has a single set of jaws, top and bottom, which unlike the hydrolysis head is free to move backwards and forwards into the A, B, and C frit positions sequentially. The jaws close around the sample frit and create an airtight chamber which is deoxygenated by the passage of argon.

Prior to derivatisation, each frit was washed with methanol containing 20 µg/ml K_3EDTA to remove any metal ions which could have interfered with the reaction, and also to dry the sample. The airtight chamber was then flushed with diisopropylethylamine (DIEA) vapour to both neutralise any acid in the sample and to facilitate coupling. Following these initial steps 5% (w/v) PITC in heptane was delivered, followed by argon to evaporate most of the heptane. A second DIEA delivery occurred to increase the polarity of the

reaction solution and to improve the coupling yields of the polar amino acids. DIEA saturated the derivatisation chamber. Coupling proceeded for 20 min at room temperature. The derivatised sample was then flushed with argon to remove most of the volatile portion of the reaction mixture. The PTC-AAs were extracted from the frit and transferred to the the transfer flask in two washings (one of 200 µl and one of 400 µl) of 0.029M sodium acetate, pH 5.00 transfer buffer. The use of aqueous buffers allowed selective extraction of the PTC-AAs, and left most of the reaction by-products behind on the frit. An argon delivery mixed the contents of the flask and then transferred the PTC-AAs to the on-line 120A PTC analyser. The entire derivatisation system was then cleaned three times with methanol, and argon dried ready for the next sample.

2.6.8 HPLC separation of PTC-amino acids

The PTC-amino acids were separated on an Applied Biosystems PTC C-18 column (5 µm particle size, 2.1 mm x 220 mm), at a flow rate of 300 µl/min at 38°C (Heinrikson and Meredith, 1984). The column was previously equilibrated with buffer A (3% (v/v) acetonitrile in 50mM sodium acetate, pH 5.40) and elution was by a gradient with 2% to 64% buffer B (70% acetonitrile in 32 mM sodium acetate, pH 6.10) over 18.8 min, then 64% to 100% buffer B over 5 min. The eluent was monitored at 254 nm. The loop size of the automatic injector was 200 µl, filled from 600 µl in the transfer flask. A standard chromatogram is shown in Fig. 2.1.

2.6.9 Difficult amino acids

Under normal hydrolysis conditions serine shows a 10-20% loss and threonine a 5-15% loss. These residues can be quantitated accurately by carrying out a time course of hydrolysis. Values for threonine and serine were therefore found by plotting the time course values (for 30, 60, and 90 min hydrolysis time) and extrapolating back to time zero.

Leucine, isoleucine and valine, when together in any combination, show slow cleavage of peptide bonds. The steric hindrance between the

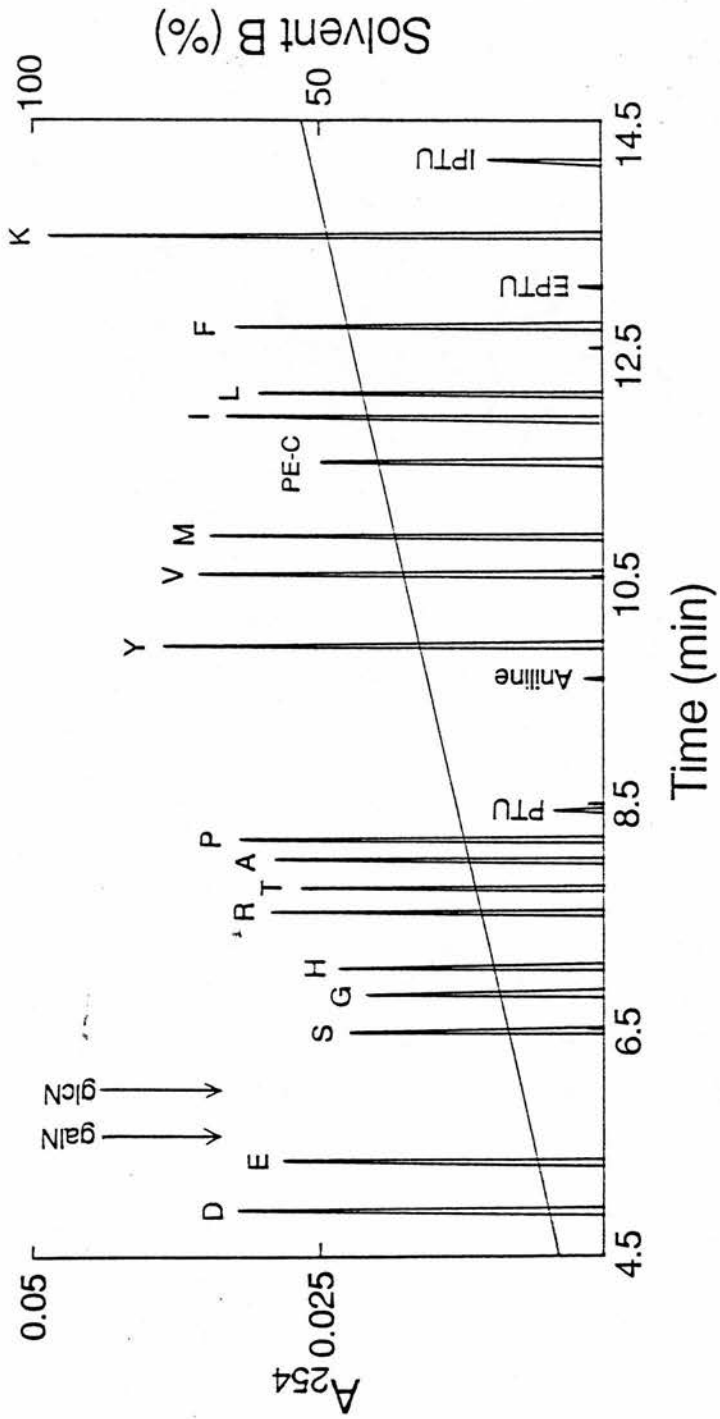


Fig. 2.1. HPLC separation of PTC-amino acids

A 50 pmol standard of 17 PTC-amino acids was separated on an Applied Biosystems PTC C-18 column (5 µm particle size; 2.1 mm x 200 mm) at a flow rate of 300 ml/min at 38°C. The column was previously equilibrated with buffer A (3% (v/v) acetonitrile in 50 mM sodium acetate, pH 5.40) and elution was by a gradient with 2% to 64% buffer B (70% acetonitrile in 32 mM sodium acetate, pH 6.10) over 18.8 min, then 64% to 100% buffer B over 5 min.

hydrophobic side chains prevents acid getting close to the bonds. The values for these residues were found by plotting the time course values and taking the values from the plateau region of maximum recovery.

It is not possible to prevent the hydrolysis of asparagine and glutamine to aspartate and glutamate respectively. Tryptophan is also completely lost on hydrolysis. The only sure way to determine amounts of these amino acids is by sequencing.

If lysine residues became carbamylated, in the presence of urea, they would form homocitruline which would be converted to lysine under acid hydrolysis. It is not possible to detect homocitruline by sequencing because of the contact with acid in the Edman degradation.

2.6.10 Pyridylethylation of cysteine residues

Cysteine is not recovered in quantitative amounts after amino acid analysis, so it is necessary to modify this residue, prior to hydrolysis, to a more stable form. Two types of methodology for determining cysteine residues were tried.

2.6.10.1 Solution phase pyridylethylation

The protein samples were dissolved in 250 µl 6M guanidine-HCl (Sigma) buffered with 0.25M Tris-HCl pH 8.5, containing 1mM EDTA. Then 10 µl 10% dithiothreitol (Sigma) was added and the solution allowed to incubate in the dark under argon at room temperature for 2h. After this time, 8 µl of undiluted 4-vinylpyridine (Aldrich) was added and the mixture incubated as above for a further 2h (Dupont *et al.,* 1987; Hawke and Yuan, 1987). Samples were then desalted on an Aquapore RP-300 C8 cartridge (7 µm particle size; 2.1mm x 30 mm) using an Applied Biosystems 130 Microbore Separation System as described previously (Section 2.4.3). It was observed that some of the reduced protein was retained by the cartridge and continued to elute in successive runs.

2.6.10.2 Vapour phase pyridylethylation

This method was found to be a simpler procedure and was preferred to the solution phase method (Amons, 1987). It also had the advantage that less pure reagents could be used as any contaminants would stay in solution. The protein after collection from a reverse phase column was dried down in a Pyrex sample tube (6 mm x 50 mm) using a Savant Speed Vac concentrator. The glass tubes had been cleaned (Section 2.6.2) and then pyrolysed at 500°C for 5h before use.

The pyridylethylation reaction was carried out in a 25 ml borosilicate glass vial (27 mm x 70 mm), containing 200 µl pyridine, 200 µl water, 40 µl 4-vinyl pyridine (Aldrich), and 40 µl tributylphosphine. After flushing with argon, the vials were sealed with PTFE/silicone sceptum, and then incubated at 60°C for 4h. The samples were then redissolved in 0.1% TFA and hydrolysed automatically on the Applied Biosystems 420A Amino Acid Analyser or hydrolysed manually at 110°C for 22h. After manual hydrolysis, residual acid and condensation were removed by placing the sample tubes into a small vacuum desiccator containing pellets of NaOH for 2 h. The samples were then redissolved in 0.025% K_3EDTA prior to amino acid analysis (Cronshaw *et al.,* 1993).

2.6.11 Quantities of proteins analysed

It was possible to purify TRAMP in large quantities. Protein concentrations that gave between 0.5 to 1.0 nmol of each amino acid, which was a generous loading, were analysed on a single frit. In the case of lysyl oxidase, which was a less stable protein, between 200 to 500 pmols of each amino acid were analysed on a single frit.

2.6.12 Calculation of amino acid compositions

To obtain higly accurate amino acid analyses, samples were analysed automatically at three hydrolysis times of 30, 60, and 90 min, and in triplicate. Threonine and serine values were found by extrapolating back to hydrolysis

time zero. Leucine, isoleucine, and valine values were found by plotting the time course values on graph paper and using the values found in the plateau region. Cysteine values were obtained by coverting cysteine to PE-cysteine in a vapour phase reaction. The values found were normalised (Ambler, 1981) to a given value for alanine. In the case of lysyl oxidase and TRAMP, alanine = 16 and 7 respectively.

2.7 Cleavage methods

2.7.1 Chemical unblocking of N-terminal pyroglutamate

Approximately 1 nmol of the TRAMP variant T3 was dissolved in 17% methylamine (Sigma) in a glass vial and incubated for 17h at 37°C (Muranova and Muranov, 1979). The methylamine was removed by desalting through an Aquapore RP-300 cartridge C8 (Section 2.4.3). The sample was then dried onto a glassfibre filter and placed into the sequencer. This procedure failed to unblock the sample, so the glassfibre filter was removed from the sequencer and exposed to 3M HCl vapour at 100°C in a special stoppered glass tube (50 mm x 10 mm), with a constriction in the middle to support the disc, for 15 min (Tsung and Fraenkel-Conrat, 1965). The filter was then replaced in the sequencer and the N-terminal sequence was found. Neither procedure on its own unblocked T3 but the combination of the two methods described above was successful.

2.7.2 Chemical unblocking of N-termini blocked with N-formyl groups

It was possible that lysyl oxidase may have been blocked by N-terminal formylation. To examine this possibility, two methods were used. In the first method approximately 200 pmol of the lysyl oxidase variant L3 was dissolved in 0.5M HCl in methanol and left at room temperature for 48h (Sheehan and Yang, 1957). The sample was dried and redissolved in 0.1% aq. (v/v) trifluroacetic acid and loaded onto the sequencer. This procedure failed to

unblock the sample. In the second method, approximately 200 pmol of L3 was loaded onto a glassfibre filter and exposed to 3M HCl at 100°C in the vapour phase, in a borosilicate glass vial (27 mm x 70 mm), for 15 min (Tsung and Fraenkel-Conrat, 1965). This method also failed to unblock the sample.

2.7.3 Pyroglutamate aminopeptidase digestion

The N-terminally blocked TRAMP variant T3 was dissolved in 240 µl buffer containing freshly prepared 0.2M NH_4HCO_3 (Sigma), 5% glycerol (BRL), 5mM EDTA (BDH) and 5mM DTT (Sigma) pH 8.0. Pyroglutamate aminopeptidase from calf liver (5-oxoprolylpeptidase, E.C.3.4.19.3; Sigma), 3 units/50 µl buffer, was added to the solution to give a final volume of 250 µl. The reaction mixture was incubated overnight at 37°C (Podell and Abraham, 1978). Desalting was accomplished by loading 200 µl of the sample onto an Aquapore RP-300 C8 cartridge (Section 2.4.3). The reduced, unblocked sample was found to be more hydrophobic than the unreduced, blocked sample as it eluted later from the reverse phase column.

2.7.4 Cyanogen bromide cleavage

Cyanogen bromide is capable of cleaving thioethers, and the stereochemistry of the methionine residue is such that proteins are cleaved specifically on the C-terminal side of methionine residues (Gross and Witkop, 1962).

An aliquot of 25 µl of a concentrated protein solution (0.1-1.0 mg/ml) was placed in a microcentrifuge tube. To this 175 µl 70% (v/v) formic acid (Sigma) was added. Working in a fume cupboard, 10 mg of CNBr was added next. The tube was covered in foil and left at room temperature for 24h. After this time 200 µl of water was added to improve the drying characteristics. The sample was dried in a Savant Speed Vac vacuum concentrator. Some 2M NaOH was placed in the cold trap to absorb any HCN present, as the NaCN formed would be less volatile. The dry sample was stored at -20°C. The sample then was redissolved in 250 µl 6M urea, 10mM phosphate, pH 7.8 immediately prior to running on the reverse phase column (Section 2.4.4).

2.7.5 Succinylation and clostripain digestion

Clostripain cleaves on the C-terminal side of arginine residues, and to a lesser extent on the C-teminal side of lysines. The latter reaction can be eliminated by succinylation of the lysine residues (Mitchell, 1977).

2.7.5.1 Succinylation

A 0.1 ml aliquot of a concentrated protein solution (0.1-1.0 mg/ml) was mixed with 0.1 ml of 2M Tris base in a microcentrifuge tube. A small amount of solid succinic anhydride (Sigma) was added every 30 min. The tubes were left at room temperature, with occasional mixing, for 4h. The succinylated protein was then dialysed against 1% NH_4HCO_3 (Sigma) for 4h with several changes (Butler *et al.,* 1969).

2.7.5.2 Clostripain digestion

Clostripain, from *Clostridium histolyticum* (Clostridiopeptidase B, E.C.3.4.22.8; Sigma) was first activated by preincubating the enzyme at a concentration of 2 mg/ml in 1mM calcium acetate (Sigma), 2mM dithiothretol (DTT; Sigma), overnight at 4°C. A 400 µl aliquot of the dialysed succinylated protein was placed in a microcentrifuge tube. (The volume increased on dialysis.) To this, 40 µl of freshly prepared 75mM DTT solution was added. After mixing, activated clostripain (5µl, or about 0.01 mg of clostripain) was added and the tube was placed in a water bath at 37°C for 4h. The sample was then stored at -20°C prior to running on a reverse phase column (Section 2.4.4).

2.7.6 Performic acid oxidation, protease V8 and endoproteinse-Asp-N digestion

Protease V8 essentially cleaves on the C-terminal side of glutamic acid when digestion is carried out in ammonium bicarbonate buffer. When digestion is carried out in sodium or potassium phosphate both glutamyl and

aspartyl bonds are cleaved. The reason for this buffer-dependent specificty is not clear (Houmard and Drapeau, 1972; Drapeau, 1977).

Endoproteinase-asp-N cleaves on the N-terminal side of aspartic and cysteic acid residues (Drapeau, 1980; Guild and Strominger, 1984).

The sample was initially oxidised with performic acid to break any cystine cross-links, as treatment of unoxidised TRAMP with protease V8 and endoproteinase-Asp-N yielded no peptides. The disadvantage of this drastic treatment, in terms of sequencing, is that cystine (and cysteine) become converted to cysteic acid which produces a blank cycle on the sequencer. Also, methionine is converted to methionine sulphone, but this can be detected on the sequencer eluting after DMPTU (Fig. 2.2).

2.7.6.1 Performic acid oxidation

Performic acid solution was prepared by adding 25 µl 30% (v/v) H_2O_2 (BDH) to 475 µl formic acid (Sigma) in a microcentrifuge tube at 4°C for 2h. The mixture was incubated at room temperature for 2h, followed by 30 min at 4°C (Hirs, 1967). TRAMP variant T3 was dissolved in 50 µl formic acid, cooled to 4°C and 100 µl ice cool performic acid solution added. The sample was incubated at 4°C for 2h. The acid was then diluted by the addition of 350 µl H_2O and the sample was dried in a Savant Speed Vac vacuum concentrator.

2.7.6.2 Protease V8 digestion

The oxidised sample was dissolved in 250 µl 100mM NH_4HCO_3 (Sigma) containing 2mM EDTA (BDH), pH 7.8. To this, 50 µl of protease V8, type XVII-B from *Staphlococcus aureus* strain V8, (Endoproteinase Glu-C, E.C.3.4.21.19; Sigma) 3 units/50 µl buffer, was added to this solution. The sample was incubated at 37°C for 5h. After this time the mixture was freeze dried. The digest was redissolved in 250 µl 6M urea containing 10mM phosphate, pH 7.8 and 200 µl loaded onto an Aquapore RP-300 C8 column (Section 2.4.3).

2.7.6.3 Endoproteinase-Asp-N digestion

The oxidised sample was dissolved in 250 µl 100 mM NH_4COOH (Sigma), pH 8.5. To this, 5 µl endoproteinase-Asp-N, Type XXXII from *Pseudomonas fragi* mutant (Sigma), 0.1 µg/5 µl buffer, was added. The sample was incubated at 37°C for 2h. After this time the mixture was freeze dried. The digest was redissolved in 250 µl 6M urea containing 10mM phosphate, pH 7.8 and 200 µl loaded onto an Aquapore RP-300 C8 column (Section 2.4.3).

2.8 Amino acid sequencing

2.8.1 Introduction

The pure peptides, in the range of 10 pmol to 2 nmol, were subject to automated sequencing on an Applied Biosystems Model 477A Microsequencer with a Model 120A on-line phenylthiohydantoin (PTH) analyser (Hayes *et al.*, 1989). Applied Biosystems chemicals were used throughout this automated analysis procedure (Section 2.8.10).

2.8.2 Sample preparation

Peptides generated by the various digests were separated by reverse phase chromatography using an Applied Biosystems Model 130A Microbore Separation System (Section 2.4.4). At this stage each peptide was pyridylethylated. This is necessary because during Edman degradation unmodified cysteine produces inconsistent results unless the reactive thiol group is chemically modified. Cysteine and cystine would otherwise produce two peaks which would elute in different places and the sequencer HPLC system is not optimised to detect these peaks. The sequencer HPLC system is optimised to detect the pyridylethylated derivative of the reduced form.

2.8.3 Vapour phase pyridylethylation of cysteine residues

All peptides were pyridylethylated prior to sequencing (Amons, 1987). The peptides were spotted onto a glassfibre filter disc and dried under a stream of nitrogen. The pyridylethylation reaction was done in a special stoppered glass tube (50 mm x 10 mm) with a constriction in the middle to support the disc. The following components, 100 µl pyridine (Sigma), 100 µl water, 20 µl 4-vinylpyridine (Aldrich) and 20 µl tributylphosphine (Sigma) were placed in the lower part of the tube. The peptide coated disc was then placed on the constriction and the tube was flushed thoroughly with argon before sealing quickly with a stopper. The vapour phase reaction was carried out at 60°C for 2h. Subsequently the disc was removed from the tube, avoiding touching any liquid, and washed for 10s in glass beakers containing 2 ml of each of the following solvents: n-heptane, n-heptane: ethyl acetate (1:1, v/v), and then ethyl acetate. The disc was then allowed to dry in air.

The disc was then wetted with 30 µl (2 mg) polybrene, a polyanion which enables the protein or peptide to bind to the disc, dried under a stream of nitrogen, and placed in the cartridge block of the Applied Biosystems Model 477A Microsequencer. To remove all traces of reagent, the sample was precycled with a wash progam (n-heptane followed by butyl chloride, 12.5% trimethylamine in water, n-heptane and ethyl acetate) before beginning the normal sequencing program. The PTH-PE-Cys derivative is resolved with the standard separation protocol used by the on-line Model 120A PTH analyser and usually elutes after PTH-Pro (Hayes *et al.*, 1989).

2.8.4 Edman degradation

The peptide loaded into the reaction cartridge was first flushed with 12% trimethylamine (TMA) to provide an alkaline environment. Then 5% phenylisothiocyanate (PITC) in heptane was delivered, followed by argon to evaporate off most of the heptane. This process was repeated three times. Coupling takes place in alkaline conditions whereby the PITC is coupled to the free uncharged N-terminal amino group of the peptide (Edman, 1967). Coupling proceeded at 48°C for 25 min. After coupling the filter disc was

washed with n-heptane followed by ethyl acetate.

Cleavage occurred by the delivery of 100% trifluoroacetic acid (TFA) to the sample. Cleavage yielded an anilinothiazolinone (ATZ) amino acid residue and a new N-terminal amino acid which could be coupled again. The sample was rinsed with ethyl acetate, soaked in n-heptane and the ATZ residue transferred with n-butyl chloride to an adjacent conversion flask.

The ATZ residue is unstable and cannot be easily identified. Therefore it was converted to the more stable phenylthiohydantoin (PTH) amino acid residue. Inside the conversion flask the ATZ residue was reacted with 25% TFA at 64°C to form the PTH-residue. The sample was taken to dryness with argon before it was redissolved in 20% acetonitrile prior to being transferred from the conversion flask to the on-line Model 120A PTH Analyser. The transfer flask was then cleaned with 20% acetonitrile, and argon dried ready for the next cycle.

2.8.5 HPLC separation of PTH-amino acids

After Edman degradation, the PTH-residues were separated on an Applied Biosystems PTH C-18 column (5 µm particle size; 2.1 mm x 220 mm) that was eluted with a 13 to 38% gradient of solvent B for 21 min followed by an isocratic elution of 38% solvent B for 7 min. Solvent A was a 1 litre aq. 5% (v/v) solution of tetrahydrofuran containing 14 ml 3M sodium acetate buffer, pH 3.8; 5.5 ml 3M sodium acetate buffer, pH 4.6; 200 µl 12.5% trimethylamine (TMA) and 35 µl acetone. Solvent B was 1 litre 100% acetonitrile containing 500 nmoles DMPTU. Chromatography was performed at 55°C using a flow rate of 210 µl/min. The eluent was monitored at 269 nm. The loop of the automatic injector was 75 µl, which was filled from 150 µl in the conversion flask. A standard chromatogram is shown in Fig. 2.2.

2.8.6 ß-Lactoglobulin-A, standard analysis

A standard of 100 pmol ß-lactoglobulin-A (ABI) was run every 3-4 months on the ABI 477A Microsequencer as a quality control test. The sequencer was set to run for 21 cycles. Within this number of cycles residues

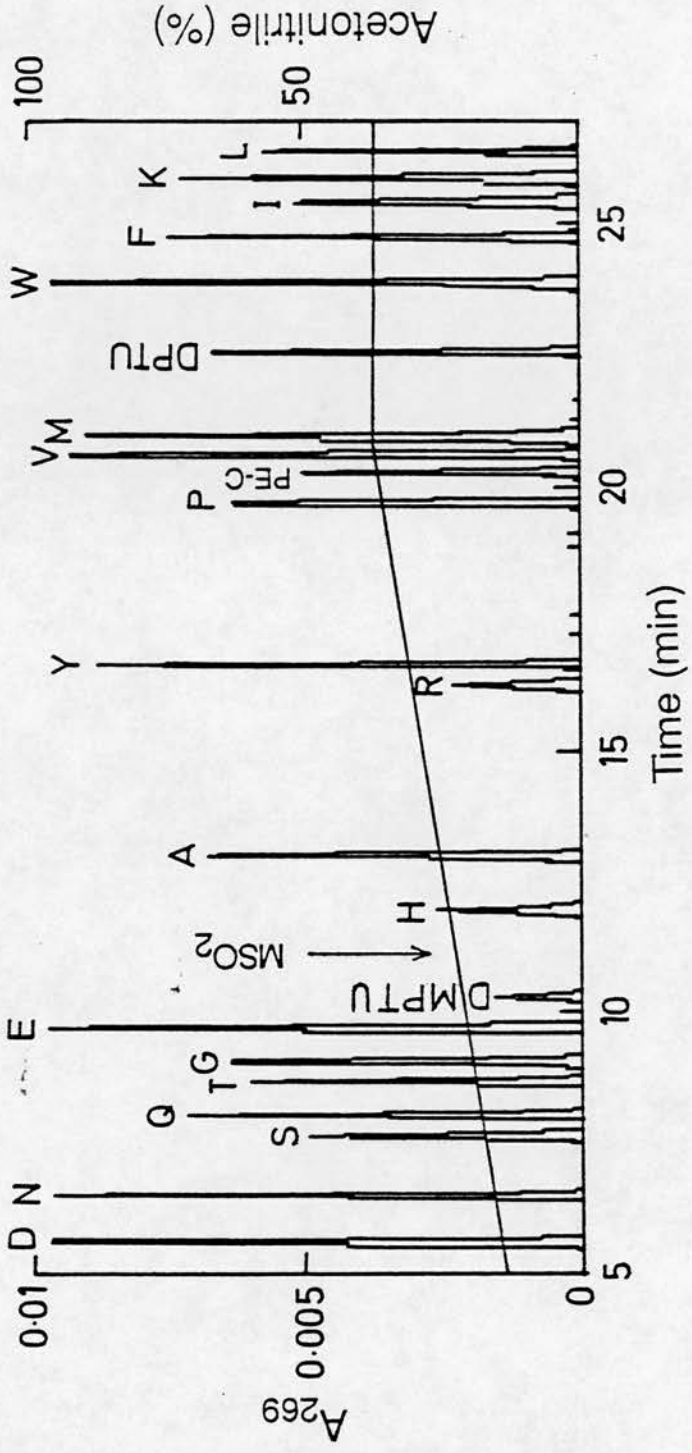


Fig. 2.2. HPLC separation of PTH-amino acids

A 75 pmol standard of 20 PTH-amino acids was separated on an Applied Biosystems PTH C-18 column (5µm particle size; 2.1 mm x 220 mm) at a flow rate of 210 µl/min at 55°C. The column was eluted with a 13 to 38% gradient of solvent B for 21 min followed by an isocratic elution of 38% solvent B for 7 min, with solvent A (aq. 5% (v/v) solution of tetrahydrofuran containing 14 ml 3M sodium acetate, pH 3.8; 5.5 ml 3M sodium acetate, pH 4.6; 200µl 12.5% TMA and 35 µl acetone) and solvent B (100% acetonitrile containing 500 nmol dimethylphenylthiourea (DMPTU)). Methionine sulphone (MSO_2) was found to elute between (DMPTU) and H, succinyllysine was found to elute between DMPTU and MSO_2.

L, I, V, T, Q, K, and G occur more than once, which enabled their individual repetitive yields, and hence a combined repetitive yield to be calculated. Repetitve yield is a measure of the efficiency of Edman degradation and is defined as:

$$\text{Repetitive Yield} = (Y_n/Y_m)^{1/n-m} \times 100$$

where Y_m = yield at cycle m, and Y_n = yield at a later cycle. The repetitive yield is best calculated between pairs of the same amino acids occurring at different positions. Combined repetitive yields calculated as the mean of the individual repetitive yields of approximately 93% were regarded as satisfactory for the successful operation of the sequencer.

2.8.7 Artefacts of Edman chemistry

The Edman reagent PITC readily reacts not only with α-amino groups in proteins, but with a wide variety of amino groups on other molecules. Some of these molecules are routinely present during protein sequencing, and several artefacts are usually observed on the HPLC chromatograms.

The most abundant artefact is diphenylthiourea (DPTU) which is formed from aniline and PITC. The aniline is derived from PITC in the presence of water and ammonia. Another artefact is dimethylphenylthiourea (DMPTU) which is derived from the reaction of PITC and dimethylamine. Dimethylamine gradually forms from trimethylamine (TMA) and for this reason TMA must be carefully stored and regularly replaced on the sequencer. A third artefact, diphenylurea (DPU) forms from diphenylthiourea in the presence of oxygen. DPTU and DMPTU are well resolved from the PTH-amino acids. However DPU is more of a problem because it co-elutes with PTH-Trp (Fig. 2.2).

2.8.8 Difficult sequences

The most common problem is probably with sequences containing proline residues. Proline differs from all other amino acids in having a side

chain that is sterically constrained into a covalent pyrridinoline ring, with the result that longer treatment with TFA is required to achieve complete cleavage at the cleavage step. Thus, during Edman degradation with standard conditions, every time a proline is encountered, the yield drops and a substantial amount of proline is detected in the next cycle. This out-of-phase sequencing persists, so that two sequences are obtained one residue out of step. If a second proline is found the problem becomes worse. Where a proline residue might be expected a special Pro cycle may be set with a longer cleavage time. This cycle cannot be used routinely as long cleavage times would cause loss of other residues such as Ser, Thr, and Trp.

Another problem is the recovery of the labile amino acids, Ser, Thr, and Trp. The PTH derivatives of these amino acids are very difficult to recover in high yield, particularly if the amount of peptide available is low.

2.8.9 Interpretation of sequence data

The traditional and still most important way of interpreting chromatograms generated by the PTH-analyser is by the eye of an experienced research worker. This is usually done by inspecting a series of cycles and looking for peaks which have increased in one cycle and decreased in the next. The sophisticated software in the computer in the 477A Microsequencer enables this analysis to be worked out automatically and printed out the sequence at the end of each run. This information was useful but manual checks of the calibration and subsequent data were absolutely essential.

2.8.10 Reagent quality

Applied Biosystems chemicals were used throughout the automatic amino acid analysis, amino acid sequencing and microbore HPLC procedures. A very high level of reagent purity was required in order to achieve high repetitive yields and high quality chromatograms. The reagents were supplied, sealed under argon, after filtration through a 0.22 μm filter.
The double distilled water used for preparing buffers for the amino acid

analyser and microbore HPLC was further purified using an Elga Elgastat UHQ water purification system. The quality of water was very important for amino acid analysis because of the high sensitivity of the instrument. Metal ion contamination in the water could cause the standard PTH-chromatogram to become distorted.

2.9 Plasma desorption mass spectrometry (PDMS)

2.9.1 Introduction

Pure peptides collected after purification by reverse phase chromatography were subject to mass analysis using an Applied Biosystems BioIon 20 Mass Analyser. Several peptides were found by reverse phase chromatography of protein digests. To facilitate sequence analysis of the peptides, preliminary mass analysis was a useful step as the sequencer could be used more efficiently by optimising the number of cycles.

Furthermore, if the initial protein sequence was known a series of mass values for the peptides could be fitted to the sequence to find the peptide of interest. The mass range measured by this instrument is from approximately 0.2K to 30K.

2.9.2 Mechanism of mass measurement using the BioIon 20 mass analyser

The BioIon 20 utilises a time-of-flight separation system. A 10 μCi Californium-252 (^{252}Cf) source was positioned behind the sample foil. The ^{252}Cf produces two fission fragments. One hits the start detector and triggers the time measurement. The other penetrates the sample foil and causes desorption of a number of secondary ions. These ions are accelerated between the foil (at a potential of 10-20 kV) and a grid (at ground potential). After the grid, the ions are allowed to drift in a field-free region (the flight tube) to the stop detector. The flight times are measured by the time-to-digital convertor (TDC). As each fission event only results in the recording of a few

ions, it is necessary to accumulate a large number of spectra. For peptides, it is necessary to record 5 x 10^5 fission events which takes 5 to 10 minutes. The flight time (T) is proportional to the mass of the ion (m) and inversely proportional to the charge of the ion (z; Macfarlane, 1990).

2.9.3 Sample preparation

Volumes of 5 to 10 µl of sample (approximately 100 pmol) from a reverse phase column were applied to a nitrocellulose-covered target. The target was spun dry to produce a monolayer of sample. The sample was then rinsed with 5 to 100 µl of purified water. If the sample had been supplied dry in a microcentrifuge tube it was dissolved in 30% (v/v) acetic acid prior to loading onto the target.

The target was then loaded into the target holder, and this was placed into an evacuation chamber inside the instrument. The target was then positioned and aligned with the Californium-252 source. The limits for detection are normally approximately 20 pmoles for peptides and approximately 1 nmole for proteins, although it may be possible to use less material (Nielsen *et al.*, 1988).

2.9.4 Calibration of the Biolon 20 mass analyser

Three peaks in the low mass region, H^+, Na^+, and NO^+, were used to calibrate the spectra. Interpretation of the plasma desorption mass spectrometer (PDMS) spectrum is usually straight forward. For small peptides (below M_r = 2,000) usually only the molecular ion (MH^+) is observed. For higher masses doubly charged ions (MH_2^{2+}) are seen and as the mass increases further, triply and higher charged species are generated. As the mass increases, the intensity between the different charged states also changes; the higher the mass the more stable is the multiply charged molecule.

2.9.5 Mixture analysis

If several peptides from a peptide digest are loaded onto one target and analysed on the PDMS, supression effects are observed, with perhaps only one peptide appearing on the spectrum. This effect is probably due to differences in hydrophobicity of different peptide components (P. Hojrup, personal communication).

PDMS analysis is not quantitative because some peptides are more readily emitted from the target than others. The precision of the PDMS is usually ± 0.1% for peptides but is sample and operator dependent. The error is around 1% when measuring proteins in the 20 to 30K region, where the instrument is working near its limit.

2.10 Matrix-assisted laser desorption mass spectroscopy (LDMS)

2.10.1 Introduction

Precise mass determinations were made with a laser desorption ion source coupled to a time-of-flight mass analyser (Lasermat, Finnigan MAT Ltd., Hemel Hempstead, U.K.). The mass range measured by this instrument is from 0.5K to 200K.

2.10.2 Mechanism of mass measurement using the Lasermat mass analyser

The Lasermat mass analyser consists of a laser desorption ion source coupled to a time-of-flight mass analyser. Light from a pulsed N_2 laser (337 nm) is focussed on to the sample target using a single fused silica lens of 50 mm focal length. The power density is adjusted to around 10^6 W/cm^2. Each pulse of desorbed ions is accelerated to 20 keV energy and directed by a cylindrical extraction lens along the axis of a 0.5 m drift tube to an electron multiplier. The digitised detector signal is then transferred to a personal computer for storage and report generation.

Spectra from multiple laser shots were averaged to improve the signal to noise ratio. All the spectra reported here were obtained in the positive ion mode. Negative (de-protonated) ions are also produced by the ionisation process, but for the majority of biological samples the positive ion spectra are generally more intense (Karas *et al.*, 1987).

2.10.3 Sample preparation

Samples (0.1-0.2 µl, approximately 6 to 10 pmol) were mixed with a 0.5 µl droplet of protein matrix (approximately 50mM sinnapinic acid in 30% (v/v) 0.1%TFA, 70% acetonitrile), in the centre of a stainless steel target and the droplet allowed to dry before introduction into the Lasermat instrument (Hillenkamp and Karas, 1990). The matrix is necessary to retain some of the protein sample in solution. Absence of matrix would lead to the protein being irreversibly adsorbed to the target surface. For the study of peptides, α-cyano-4-hydroxycinnamic acid was used in preference to sinnapinic acid as it showed fewer matrix interference peaks in the lower mass region of the spectra (Beavis *et al.*, 1992).

2.10.4 Calibration of the Lasermat mass analyser

Samples of lysyl oxidase and TRAMP variants were calibrated using carbonic anhydrase (M_r = 29,024) as external standard. A sample of carbonic anhydrase was run on a sample slide and its calibration constants (required for converting time to mass), were stored and used for subsequent sample analysis (Karas and Hillenkamp, 1988). Small molecular weight peptides need a separate calibration. Substance P (synthetic porcine; M_r = 1,347.7) and renin substrate (porcine; M_r = 1,759) were used as external standards to measure the masses of lysyl oxidase CNBr and endoproteinase Asp-N peptides.

Molecular masses measured by this technique correspond to average (or chemical) molecular mass of the protonated species. Calculated values used for comparison are derived from IUPAC average atomic masses (C = 12.011, H = 1.008, N = 14.007, and O = 15.999).

2.11 Chemical deglycosylation

Chemical deglycosylation was used in the analysis of some samples (Sojar and Bahl, 1987). For this, approximately 1 nmol protein was dried in a microcentrifuge tube. To which 150 µl anhydrous trifluoromethanesulphonic acid (TFMS; Aldrich) was added. The reaction mixture was incubated at 0°C under argon for 2h. N-glycoside links require a short incubation time while O-glycoside links, which are more stable, require a longer incubation period. The 2h incubation period was selected to enable both types of linkage, if present, to be cleaved. Susequently the reaction mixture was cooled to -20°C by placing the tube in dry ice-methanol contained in a vacuum flask, and then neutralised by the addition of 60% pyridine (also cooled to -20°C). The temperature must be carefully controlled since pyridine-TFMS salt formation is a strongly exothermic reaction. The neutralised reaction mixture was dialysed at 4°C against several changes of 0.01% NH_4HCO_3. The sample was then applied to a microbore HPLC system with a RP-300 C8 cartridge and eluted with a linear gradient to 70% (v/v) acetonitrile (Section 2.4.3). The eluted protein was collected and dried using a Savant Speed Vac vacuum concentrator for Lasermat mass analysis.

2.12 Desulphation

For desulphation, approximately 1 nmol TRAMP was dialysed into 0.1M sodium acetate pH 5.0, then 0.1 to 0.25 units of aryl-sulphatase (Aryl-sulphate sulphohydrolase from *Helix pomatia,* EC 3.1.6.1; Boehringer) were added in a total volume of 0.2 ml. The reaction mixture was incubated for 2h at 37°C. After this time 80 µl was removed and immediately heated to 100°C for 2 min in SDS-PAGE sample buffer. To the remainder of the sample 120 µl 6M PBU was added and the sample stored at -20°C (Dodgson and Spencer, 1953).

2.13 Determination of sulphotyrosine by alkaline hydrolysis

2.13.1 Chemical synthesis of sulphotyrosine

Sulphotyrosine was synthesised (by J.R.E. MacBeath) by reaction of L-tyrosine with concentrated sulphuric acid at low temperatures (Reitz *et al.*, 1946). Prior to mixing, 5g tyrosine and 10 ml concentrated sulphuric acid were separately cooled in dry ice (-10 to -25°C) for 15 min. The reagent were then mixed and stirred rapidly over ice for 10 min, and then neutralised with $Ba(OH)_2$. The sulphotyrosine was freed from unreacted tyrosine and Ba^{2+} ions by passage through a Bio-Rad AG 50W-X8 (200-400 mesh, H^+ form) cation exchange column. Sulphotyrosine, Ba^{2+} salt is stable at -20°C for at least a year (Huttner, 1984).

2.13.2 Measurement of sulphotyrosine by alkaline hydrolysis

The ester bond of sulphotyrosine is labile in acid but stable in alkali (Huttner, 1984). Therefore, under acid hydrolysis and protein sequencing conditions any sulphotyrosine that may be present would be converted to tyrosine. In view of this TRAMP was subjected to alkaline hydrolysis.

Approximately 500 pmol TRAMP was placed into a pyrolysed Pyrex sample tube (6mm x 50 mm). Then 200 µl 0.2M $Ba(OH)_2$ was added to the tube and the tube placed in the bottom of the glass vial, then sealed with a Teflon/silicone septum and screw cap. The vial was placed in an oven at 110°C for 22h. After hydrolysis the sample was neutralised with approximately 25 µl 1M H_2SO_4. The hydrolysate was transferred to a microcentrifuge tube and the precipitate pelleted by centrifugation in an IEC-Centra-M-Microcentrifuge at 15,600 g for 5 min. The supernatant was decanted for subsequent high voltage paper electrophoresis (Section 2.5.10) or amino acid analysis (Section 2.6). Sulphotyrosine was found to elute between Pro and PTU (Fig. 2.1) on a PTC C-18 column. Sulphotyrosine was also found to separate well from tyrosine by high voltage paper electrophoresis using pH 6.5 buffer (Fig. 3.31, Section 3.3.8).

2.14 Cell culture and immunoprecipitation of [^{3}H]- and [^{35}S]-labelled TRAMP

2.14.1 Introduction

The biosynthesis of TRAMP was investigated in cell culture labelled with [3,5-^{3}H]tyrosine or ^{35}S-sulphate. Using an anti-TRAMP antibody (provided by E. Forbes) radiolabelled TRAMP was immunoprecipitated from both culture media and cell lysates, and then analysed by SDS-PAGE. Following electroelution or electroblotting and alkaline hydrolysis, radiolabelled tyrosine and sulphotyrosine were analysed by HVPE or by amino acid analysis.

2.14.2 Cell culture

Human foreskin fibroblasts (passage 7) were obtained (from G.W. Preistley, Dept. of Dermatology, University of Edinburgh) and stored under liquid nitrogen. For cell culture (Freshney, 1986) a vial containing 3 x 10^{6} cells was thawed quickly at 37°C and washed with 10 ml Dulbecco's Modified Eagle's Medium (DMEM), 10% (v/v) foetal calf serum, 100 IU/ml penicillin, 100µg/ml streptomyocin, in a 15 ml sterile polystyrene graduated conical tube. After centrifugation at 1,000 g for 3 min, the cells were resuspended in 10 ml DMEM. Two aliquots of 5 ml of resuspended cells were placed in each of two polystyrene 75 cm^{2} cell culture flasks containing 15 ml DMEM. The plastic tops on the culture flasks were opened by one full turn, to allow gas exchange, and placed in a Grant CO_2 cell culture incubator. Cells were incubated at 37°C in an atmosphere of 5% CO_2, and the medium was changed (to remove traces of DMSO left from liquid nitrogen storage) after 24h. When the cells had reached confluency (after aproximately 5 days) they were harvested using trypsin/EDTA (by D.J.S. Hulmes) and then plated out into ten cell-culture flasks and incubated for a further four days. At confluency, the medium was replaced by serum-free Minimal Essential Medium (MEM), prepared (from a GIBCO Select Amine Kit) either without tyrosine or free of sulphate ($MgSO_4$ and $CaSO_4$ replaced by $MgCl_2$ and $CaCl_2$) and with the

corresponding radiolabel, either [3,5-^{3}H]tyrosine (5 flasks) or ^{35}S-sulphate (5 flasks) respectively. Each medium was filtered through a 500ml disposable 0.2 μm bottle filter (Costar) prior to addition to the cells. The radiolabels, from stock solutions in dilute HCl, were first dried in a Savant Speed Vac vacuum concentrator, then dissolved in appropriate tyrosine-free or sulphate-free medium) were used at final concentrations of 50 μCi/ml ([^{3}H]tyrosine) or 100 μCi/ml (^{35}S-sulphate), respectively. Cells were incubated in the presence of radiolabel (10 ml medium per 75 cm^{2} flask) for approximately 24h.

Following incubation, the culture medium was collected and pooled to give a total volume of 50 ml, placed on ice and proteinase inhibitors were added to final concentrations of 20mM EDTA, 10 mM N-ethylmaleimide (NEM), 1 ml phenylmethylsulphonylfluoride (PMSF) in 50 mM Tris-HCl, pH 7.5. The medium was spun at 10,000 g for 1 min and the supernatant stored at -20°C. The cell layers were lysed with 1 ml (per flask) of lysis buffer (1% Nonidet NP-40, 0.15M NaCl, 20 mM EDTA, 10 mM NEM, 1 ml PMSF, 50 mM Tris-HCl, pH 7.50) at 4°C for 30 min. The cell layer was then released with a cell scraper, and the lysate removed using a pipette. The flasks were subsequently washed with 1.5 ml lysis buffer to give a final lysate volume of 6.5 ml. The combined extracts were centrifuged, at 10,000 g at 4°C for 10 min, followed by separation of supernatant (cell lysate) and pellet.

2.14.3 Immunoprecipitation

Proteins were immunoprecipitated, from culture medium or from cell lysates, using killed *Staphylococcus aureus* bacteria (*Staph. A.*; Calbiochem) that were preloaded either with pre-immune serum or with anti-TRAMP anti-serum (Harlow and Lane, 1988). The *Staph. A.* were centrifuged at 10,000 g for 1 min and resuspended twice in buffer A (9 parts MEM: 1 part 500mM Tris-HCl, 200mM EDTA, pH 7.5). Then 500 μl *Staph. A.* were incubated at 4°C for 30 min with 50 μl pre-immune serum (PIS). The PIS-loaded *Staph. A.* were then washed three times by centrifugation at 10,000 g for 1 min and resuspension in buffer A, to a final volume of 250 μl.

To the cell culture medium and cell lysates, bovine serum albumin (BSA; 1 mg/ml in buffer A) was added to a final concentration of 100 μg/ml.

BSA was added, to reduce non-specific binding of the antibody to other proteins. The PIS-loaded *Staph. A.* (50 µl) were then added to the medium (1ml), or to the lysate (100 µl), followed by incubation with gentle agitation at 4°C for 2h. Proteins bound non-specifically to the PIS-loaded *Staph. A.* were then removed by centrifugation at 10,000 g for 1 min and the supernatants were retained for immunoprecipitation with *Staph. A.* pre-loaded with anti-TRAMP anti-serum. The pellets were extracted in 250 µl SDS-PAGE sample buffer (2% (w/v) SDS, 0.125M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 3% (v/v) 2-mercaptomethanol and 0.005% (w/v) bromophenol blue) at 100°C for 3 min, followed by centrifugation at 10,000 g for 1 min. The supernatants were stored at -20°C prior to SDS-PAGE. *Staph. A.* were pre-loaded with anti-TRAMP anti-serum as for the pre-immune serum, and further incubations, centrifugations and resuspensions were as above, except that supernatants following pre-incubation of lysates and media with PIS-loaded *Staph. A.* were used.

2.15 [^{14}C]-labelling of purified TRAMP and molecular weight standards

TRAMP and molecular weight standards (Pharmacia) were radiolabelled (by D.J.S. Hulmes) by reductive alkylation with [^{14}C]formaldehyde and cyanoborohydride (Dottavio-Martin and Ravel, 1978). TRAMP (5 mg/ml), previously in 6M PBU, was resuspended in 0.04M potassium phosphate buffer, pH 7.0, using Amicon C10 concentrators. To 125 µl of a 1mg/ml solution of TRAMP in 0.04M potassium phosphate buffer, pH 7.0, 50 µl [^{14}C]formaldehyde (500 µCi/ml) was added followed by 50 µl of a freshly prepared solution of $NaBH_3CN$ (6 mg/ml) in 0.04M potassium phosphate buffer. The reaction mixture was then incubated, with agitation, at 25°C for 3h. Unincorporated label was then removed using an Amicon C-10 microconcentrator, using several changes of 0.04M potassium phosphate buffer. The same procedure was used for [^{14}C]-labelling of the molecular weight markers.

2.16 Fluorography

Fluorography (Bonner and Laskey, 1974) was used to detect the labelled proteins. Directly after electrophoresis the SDS-PAGE gel was soaked in 200 ml dimethylsulphoxide (DMSO) for 20 min, followed by a second immersion in fresh DMSO for a further 20 min. The gel was then soaked in 100 ml 20% (w/v) 2,5-diphenyloxazole (PPO) in DMSO for 1.5h. The PPO acts as a fluor increasing the radioactive signal intensity by approximately four fold. PPO was then precipitated in the gel by immersing in 200 ml water for 1h. In order to prevent the gel from cracking on drying, the gel was soaked in 10% methanol containing 0.1% glycerol. The gel was dried on to Whatman 3MM paper under vacuum and placed in contact with Kodak X-OMAT AR X-ray film and exposed at -70°C for 4-72h. Exposure at -70°C produces a more discrete, less diffuse image on the film. At lower temperatures the neutrons from low β-emitting sources, such as ^{14}C and ^{35}S, have less energy and travel shorter distances. After exposure the film was developed using an X-OGRAPH COMPACT-XZ automatic developer.

2.17 Electroelution

Electroelution of TRAMP from SDS-PAGE bands was performed in an electroelution apparatus containing 25mM Tris-base, 192mM glycine and 0.025% SDS, pH 11.0 (Jacobs and Clad, 1986). Protein migrated from the bands in contact with the cathode buffer reservoir, to the protein trap, in contact with the anode buffer reservoir. After elution at constant 200V for 3h, the electroeluate was collected from the protein trap for further analysis.

2.18 Electroblotting

The SDS-PAGE gel was electroblotted onto a ProBlott membrane (ABI; Matsudaira, 1987). After electrophoresis the gel was transferred to CAPS buffer (10mM 3-[cyclohexylamino]-2-hydroxy-1-propanesulphonic acid

(CAPS), 10% methanol, pH 11.0) and left for 5 min to reduce the amount of Tris and glycine. The ProBlott membrane was cut to the same size as the gel and rinsed in 100% methanol for 2-3s and then equilibrated in CAPS buffer. A gel and ProBlott sandwich was made between three layers of Whatman 3MM paper and assembled in a semi-dri blotting unit. Electrophoretic transfer was carried out at a constant current of 0.8 mA/cm^2 (approximately 90 mV) for 1h. Afterwards the membrane was removed, rinsed in water for 5 min, stained (0.1% Coomassie Blue in 50% methanol) for 5 min, and then destained (10% acetic acid, 50% methanol) for 10 min. The blot was left to dry and the relevant bands cut out for alkaline hydrolysis or other procedures. Unlabelled TRAMP standard was added to the radiolabelled immunoprecipitates to facilitate identification of bands on ProBlott.

Chapter 3 Results

3.1 Purification of lysyl oxidase and TRAMP

Lysyl oxidase was extracted from porcine skin following the scheme shown in Fig. 3.1 (Section 2.3; Shackleton and Hulmes, 1990a). The crude extract supernatant (E) was diluted to 2M PBU and loaded onto a CM-Sepharose Fast Flow column previously equilibrated with 2M PBU. The flow-through solution was then immediately applied to a DEAE-Sepharose Fast Flow column, previously equilibrated with 2M PBU. The column was then washed with 2 column volumes of 2M PBU, followed by 2 column volumes of PB and then most of the bound proteins were eluted (but not lysyl oxidase or TRAMP) with PB containing 0.3M NaCl (Fig. 3.2a). To release bound proteins containing lysyl oxidase activity but relatively uncontaminated with TRAMP, the column was then eluted with 3M PBU containing 0.3M NaCl. Fractions were assayed for lysyl oxidase activity. The activity was completely inhibited by 0.2 mM ßAPN, and active fractions were pooled as DEAE pool 1 (Fig. 3.2b). A subsequent elution with 6M PBU containing 0.5M NaCl released, predominantly, TRAMP. The fractions containing TRAMP were collected as DEAE pool 2 (Fig. 3.2b). Lysyl oxidase and TRAMP were then purified separately.

The DEAE pool 1 was passed through a Sephadex G-25 gel filtration column, previously equilibrated with PB, to remove the urea and salt. The eluted protein was collected and pooled as G-25 pool 1(Fig. 3.3a). The DEAE pool 1 (600ml) was loaded in two stages in order not to saturate the G-25 column. The second loading was collected as G-25 pool 2 (Fig. 3.3b). These two pools were added together to form G-25 pool 1+2. The DEAE pool 2 was desalted likewise and collected as G-25 pool 3 (Fig. 3.3c).

The G-25 pool 1+2 was then loaded onto a Sephacryl S-400 column previously equilibrated with PB. The column was washed with two column volumes of PB. Bound proteins were eluted first with 1.5M PBU, followed by 6M PBU (Fig. 3.4a). Lysyl oxidase appeared in the 6M PBU eluate. Individual fractions were assayed before being collected together as S-400 pool 1.

To further purify TRAMP, the G-25 pool 3 was loaded onto the Sephacryl S-400 column previously equilibrated with PB. The column was washed with 1.5M PBU, followed by 6M PBU (Fig. 3.4b). TRAMP appeared in

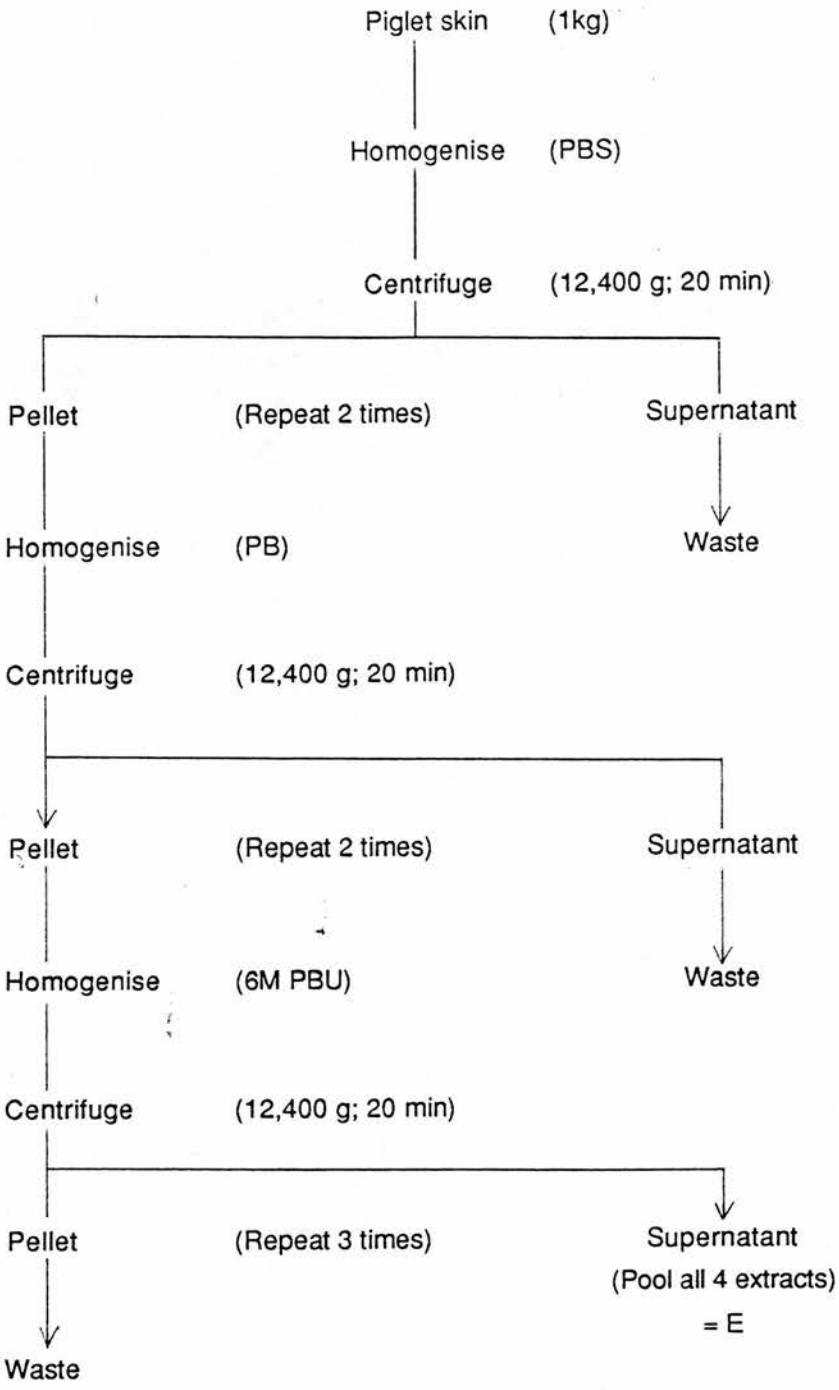


Fig. 3.1. Preparation of an extract of lysyl oxidase and TRAMP

This diagram shows an outline summary of the extraction procedure described in Section 2.3.4. (Shackleton and Hulmes, 1990a).

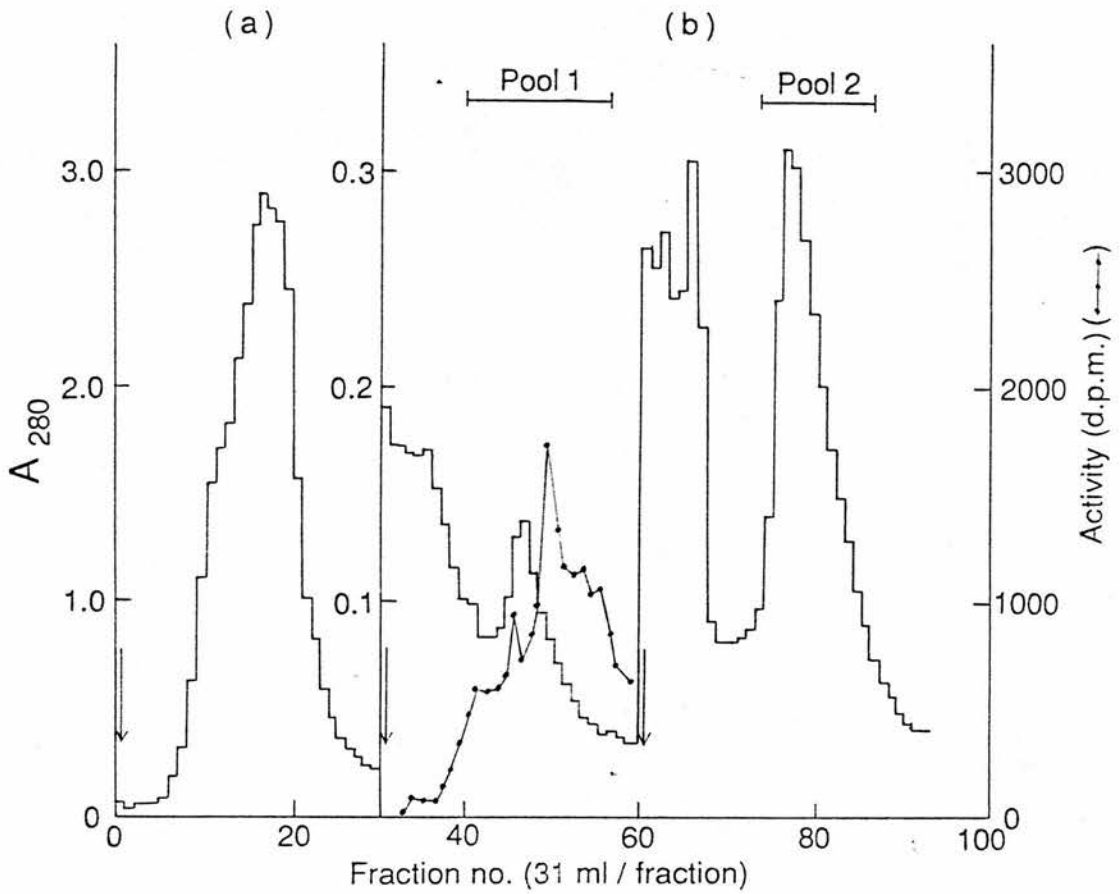


Fig. 3.2. Separation of piglet skin extract by DEAE-Sepharose chromatography

(a) The CM-Sepharose flow through solution was loaded onto the DEAE-Sepharose column (5 cm x 25 cm; volume = 500 ml) at a flow rate of 30 ml/min. The arrow shows the start of elution of the DEAE-Sepharose column with 0.3M NaCl. The protein peak contains a mixture of unwanted proteins. Lysyl oxidase and TRAMP remained bound to the column.

(b) The first arrow shows the start of elution of the DEAE-Sepharose column with 3M PBU containing 0.3M NaCl. The fractions shown to have lysyl oxidase activity were collected as DEAE pool 1. The second arrow shows the start of elution of this column with 6M PBU containing 0.5M NaCl. The first peak to elute contained a mixture of unwanted proteins excluding lysyl oxidase and TRAMP. The second peak containing TRAMP was collected as DEAE pool 2.

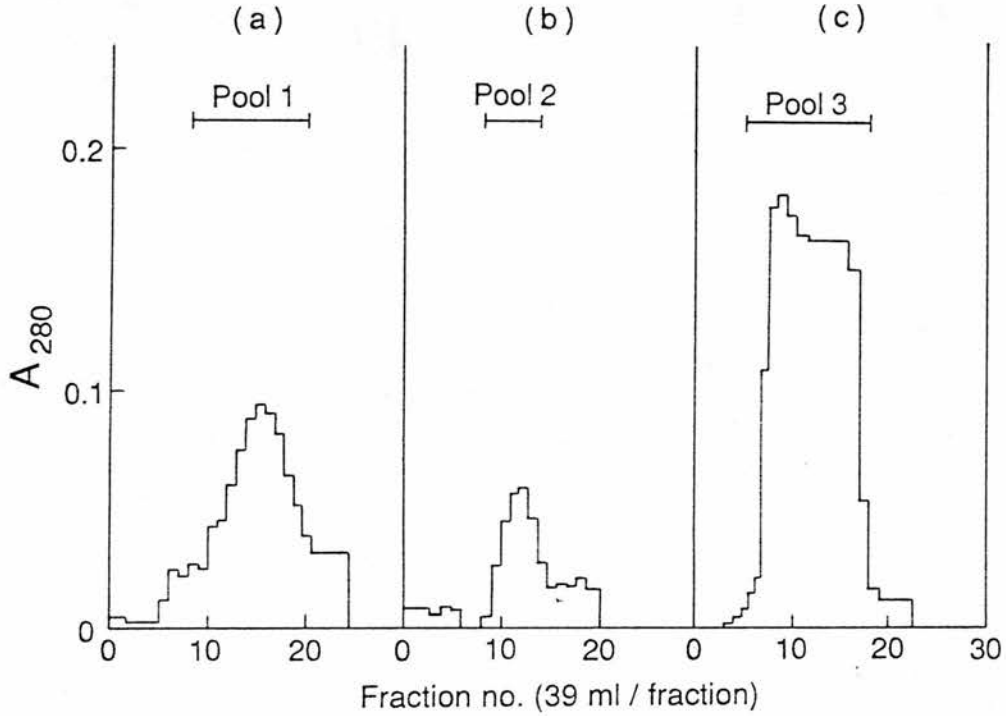


Fig. 3.3. Desalting of lysyl oxidase and TRAMP pools from the DEAE-Sepharose column using Sephadex G-25 chromatography

(a) 400 ml of the DEAE pool 1 were loaded onto the Sephadex G-25 column (5 cm x 100 cm, volume = 2 litre), at flow rate of 40 ml/min, previously equilibrated with PB and then eluted with PB. The desalted lysyl oxidase was collected as G-25 pool 1.

(b) The remaining 200 ml of the DEAE pool 1 were desalted as described above. The desalted lysyl oxidase was collected as G-25 pool 2, and G-25 pool 1 and 2 combined. The maximum volume that this column could desalt at a time was 400 ml, hence the DEAE-Sepharose lysyl oxidase pool 1 was loaded in two stages.

(c) 400ml of DEAE pool 2 were loaded onto the Sephadex G-25 column as described above. The desalted TRAMP was collected as G-25 pool 3.

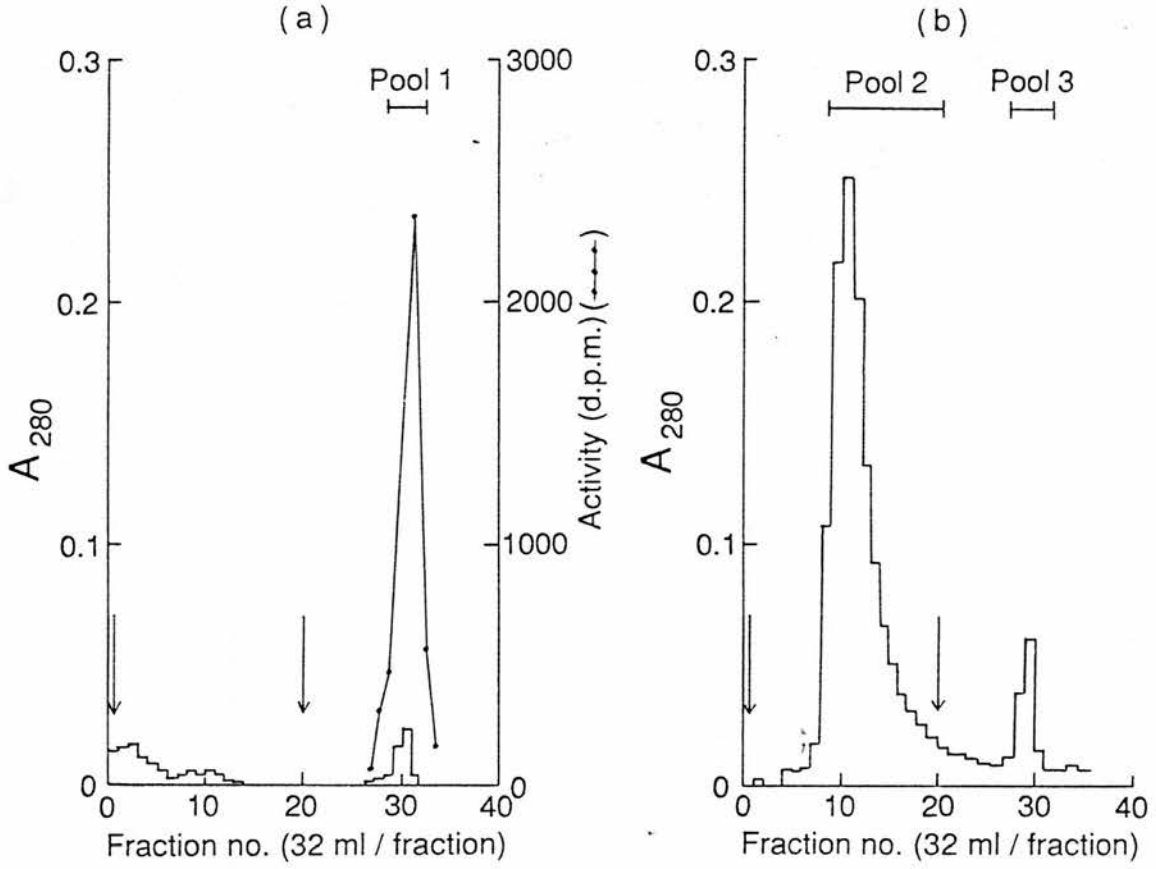


Fig. 3.4. Separation of lysyl oxidase and TRAMP by Sephacryl S-400 "affinity" chromatography

(a) The Sephadex G-25 pools 1 and 2 were combined and loaded onto a Sephacryl S-400 column (5 cm x 20 cm; volume = 400 ml), previously equilibrated with PB at a flow rate of 15 ml/min. The column was then eluted with 1.5M PBU, starting at the first arrow, to remove any unwanted TRAMP followed by 6M PBU, starting at the second arrow. The fraction showing lysyl oxidase activity was collected as S-400 pool 1. This activity was completely inhibited by 0.2 mM βAPN.

(b) The Sephadex G-25 pool 3 was loaded onto the Sephacryl S-400 column previously equilibrated with PB. The column was then eluted with 1.5M PBU, shown by the first arrow. The TRAMP which eluted was collected as S-400 pool 2. The column was then eluted with 6M PBU, shown by the second arrow. The protein peak, containing a mixture of lysyl oxidase and TRAMP, was collected as S-400 pool 3.

the 1.5M PBU eluate and was collected as S-400 pool 2. A mixture of lysyl oxidase and TRAMP was found in the 6M PBU eluate which was collected as S-400 pool 3.

Aliquots of the crude extract (E, diluted 1:3) DEAE pool 1, DEAE pool 2, S-400 pool 1, S-400 pool 2, and S-400 pool 3 were run on a 12% SDS-PAGE gel (Fig. 3.5). The highly purified lysyl oxidase in S-400 pool 1 and TRAMP in S-400 pool 2 were used for further study. Details of the recoveries of lysyl oxidase from this purification procedure are shown in Table 3.1.

3.2 Lysyl oxidase

3.2.1 Separation of enzyme variants

Lysyl oxidase purified by Sephacryl S-400 chromatography (S-400 pool 1; Fig. 3.4a) was further purified by Mono Q anion exchange FPLC, (Fig. 3.6a). Four variants of lysyl oxidase (L1 to L4) were resolved, with small amounts of TRAMP variants also present. Peaks from the Mono Q column were analysed by SDS-PAGE (Fig. 3.7), and the electrophoretic migration of the four lysyl oxidase variants was found to be identical. Each variant was also assayed for lysyl oxidase activity (Section 2.1; Table 3.2) and there appeared to be very little difference in specific activity between the four variants. Similarly, all four variants of lysyl oxidase eluted at the same position by reverse phase HPLC (Fig. 3.8). Lysyl oxidase was found to have a shorter retention time than TRAMP, which therefore enabled the two proteins to be separated. Reverse phase chromatography was also used to desalt the proteins prior to further structural and compositional analysis.

3.2.2 Chromatofocussing

A Mono P chromatofocussing column (Section 2.4.5) was equilibrated with buffer A (0.025M Bis-Tris, pH 4.70). The lysyl oxidase sample (S-400 pool 1) in 6M PBU was applied to the column. When the pH was steady, buffer B (Pharmacia Polybuffer 74, pH 3.15) was applied to the column, as shown

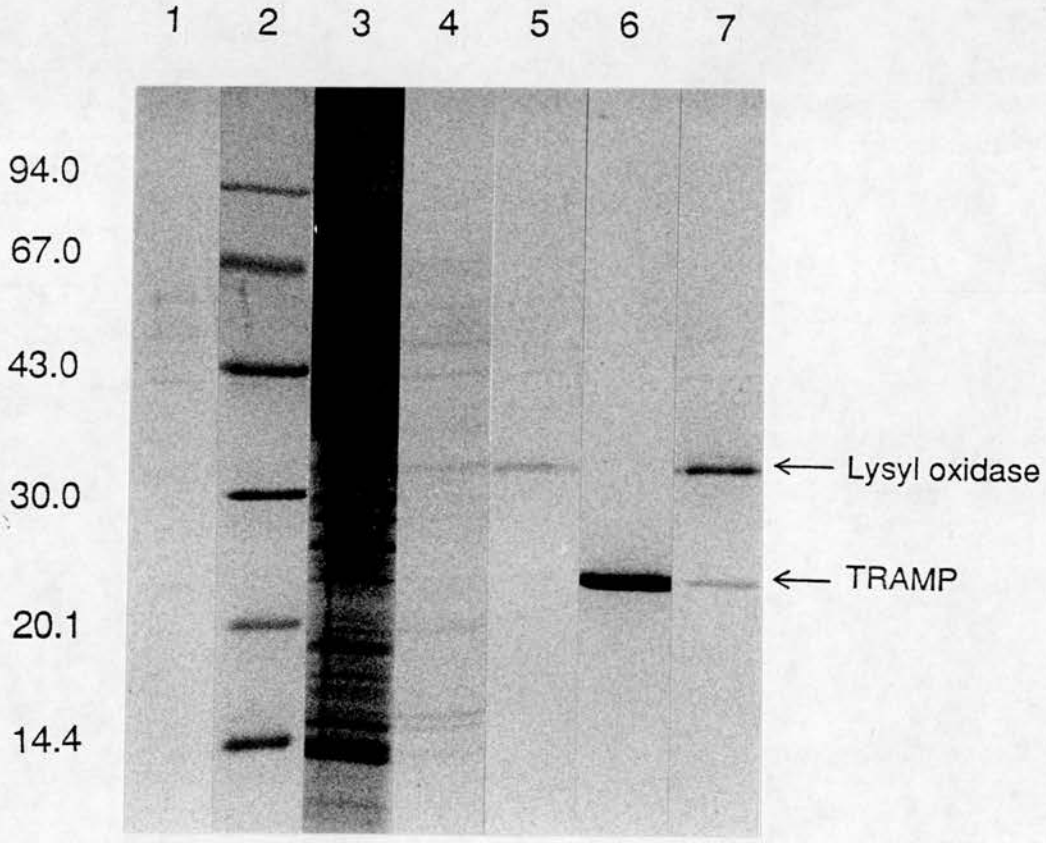


Fig. 3.5. SDS-PAGE analysis of the different stages of purification of lysyl oxidase and TRAMP

Proteins were analysed on a 12% SDS-PAGE gel (in reducing conditions) and stained with Coomassie Blue. The lanes are: (1) Blank, showing some high molecular bands due to contamination from 2-mercaptoethanol present in the sample buffer, (2) protein standard, (3) initial 6M PBU extract E, diluted 1: 3, (4) DEAE-elution containing lysyl oxidase, (Fig. 3.2, pool 1), (5) S-400 elution of lysyl oxidase, (Fig. 3.4, pool 1), (6) S-400 elution of TRAMP, (Fig. 3.4, pool 2), (7) S-400 elution of scavenged lysyl oxidase and TRAMP, (Fig. 3.4, pool 3). The molecular masses (K) of protein standards are indicated.

Table 3.1. Purification of lysyl oxidase from 1kg piglet skins

Purification Step	Total protein (mg)	10^{-6} Total activity (d.p.m.)	Specific activty (d.p.m./µg)	Yield (%)	Purification (fold)	Inhibition with 0.2µM βAPN (%)
6M PBU extract	6.53×10^3	142.3	22	100.0	1	99
DEAE-Sepharose	33.48	36.9	1102	25.9	50	95
S-400 pool 1	0.76	15.1	19945	10.6	907	98

Protein concentrations were measured by the Bradford (1976) method, with bovine serum albumin (BSA) standards.

Table 3.2. Assay of the four lysyl oxidase variants

Protein	Total protein (µg)	Total activity (d.p.m.)	Specific activity. (d.p.m./µg)	Protein (% of total recovered)
S-400 pool 1	950	22,091,000	23,300	
L 1	15	366,000	24,400	11.1
L 2	32	705,000	22,000	21.1
L 3	72	1,686,000	23,400	51.4
L 4	21	533,000	25,400	16.2
Total recovered	140	3,290,000		
Yield	14.7%	14.9%		

Protein concentrations were measured by the micro BCA protein assay (Smith *et al.* 1985), with BSA standards. S-400 pool1 = lysyl oxidase starting material prior to Mono Q anion exchange chromatography i.e. pool 1 Fig. 3.4a. Lysyl oxidase was assayed by measurement of tritiated water release from tritiated lathyritic elastin (Shackleton and Hulmes, 1990b). The low yields are possibly due to permanent binding of lysyl oxidase to the Mono Q column.

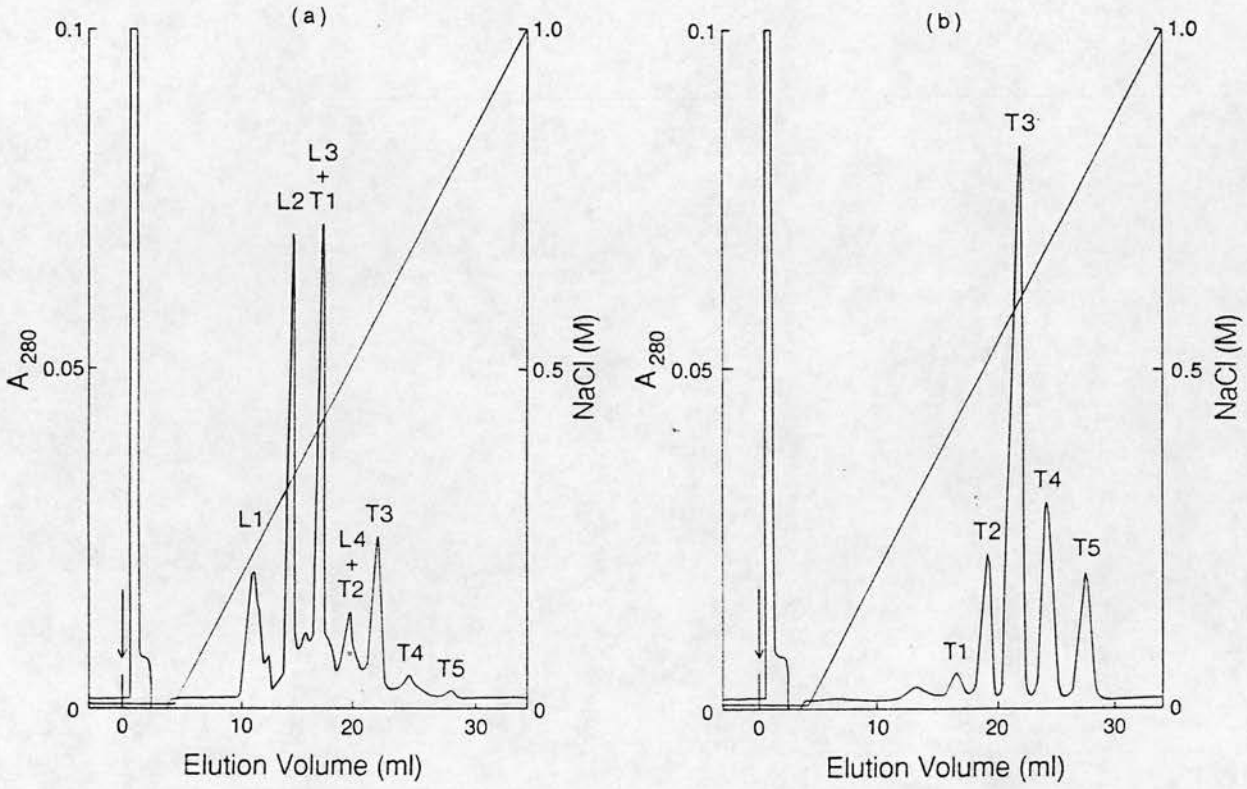


Fig. 3.6. Separation of four variants of lysyl oxidase and five variants of TRAMP by anion exchange FPLC

Lysyl oxidase and TRAMP extracts, following purification by Sephacryl S-400 chromatography (Fig. 3.4a, pool 1 and Fig. 3.4b, pool 2, respectively) were further purified on a Pharmacia Mono Q HR 5/5 column. (a) Lysyl oxidase variants L1 to L4, with small amounts of TRAMP variants T1 to T5. (b) TRAMP variants T1 to T5.

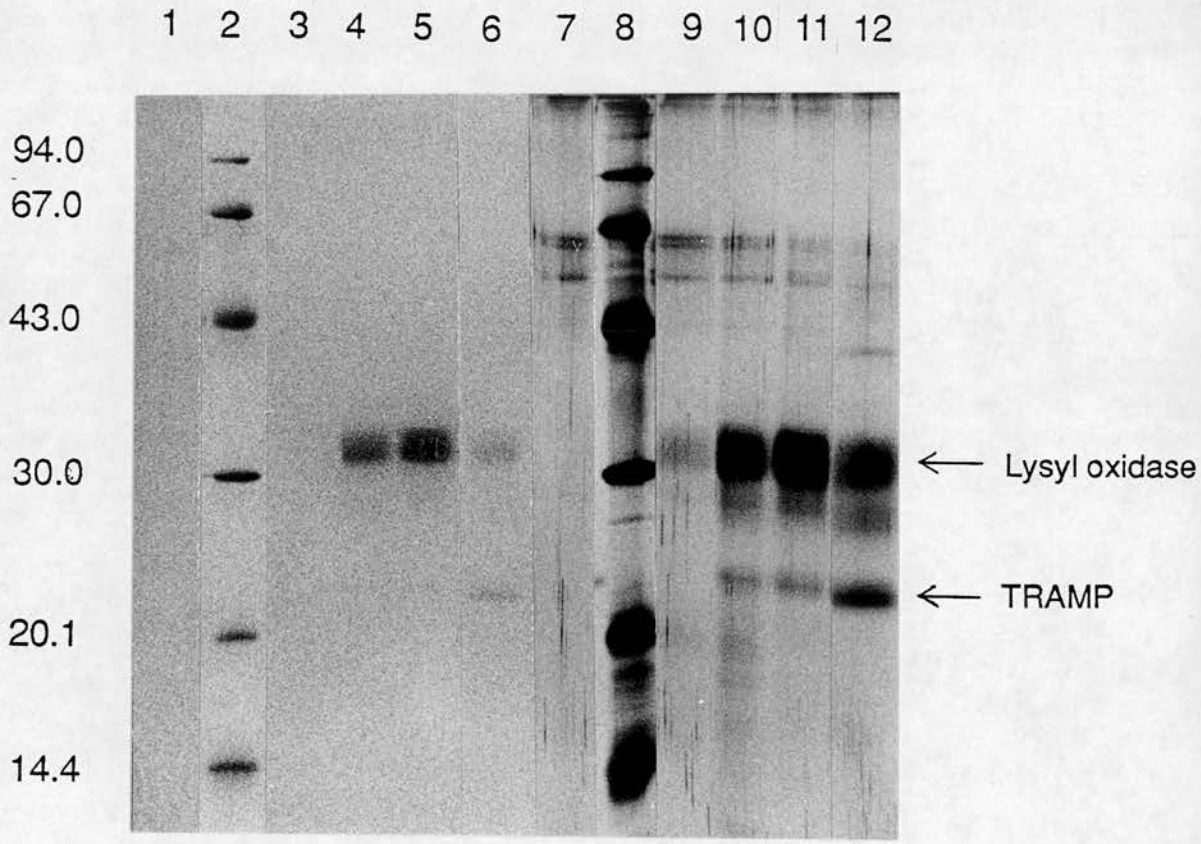


Fig. 3.7. SDS-PAGE analysis of four variants of lysyl oxidase after anion exchange chromatography

Lysyl oxidase variants L1 to L4 with small amounts of TRAMP variants. The lanes are: (1) blank, (2) protein standards, (3) L1, (4) L2, (5) L3, (6) L4, (7) blank, (8) protein standards, (9) L1, (10) L2, (11) L3, (12) L4. Lanes (1-6) were stained with Comassie Blue, whilst lanes (7-12) were stained with silver stain. Approximately 1μg of protein was loaded in each lane and analysed (in reducing conditions) on a 12% SDS-PAGE gel. The molecular masses (K) of protein standards are indicated.

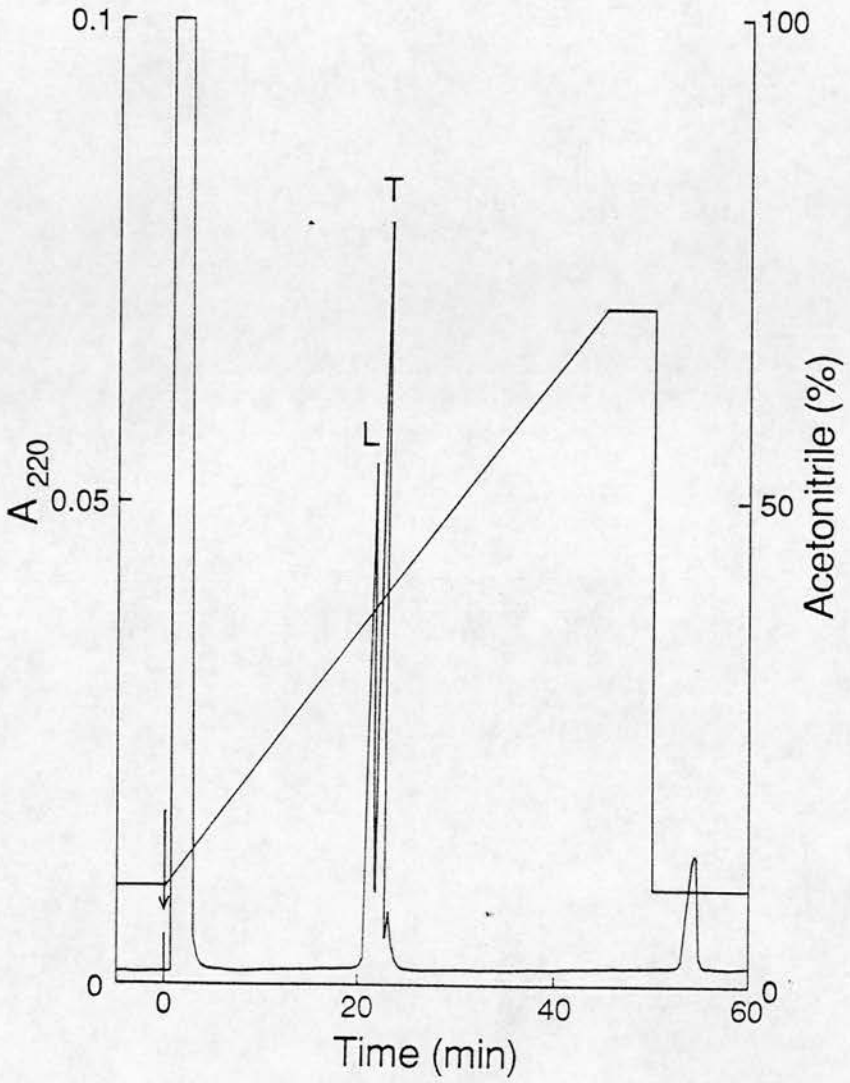


Fig. 3.8. Separation of lysyl oxidase (L4) from TRAMP (T2) by reverse phase chromatography

The different fractions of lysyl oxidase and TRAMP collected from the Mono Q HR 5/5 anion exchange column were individually loaded onto a Aquapore RP-300 C8 reverse phase cartridge (7 µm particle size; 2.1 mm x 30 mm) at a flow rate of 100 µl/min. Lysyl oxidase was found to have a shorter retention time than TRAMP enabling the two proteins to be separated and collected for subsequent analysis. Preparative amounts of sample were separated on a Pharmacia ProRPC HR 5/2 (C1/C8) reverse phase column (5 µm particle size; 5 mm x 20 mm) by applying the same gradient as above with a flow rate of 2 ml/min. Lysyl oxidase also eluted before TRAMP under these conditions. All four variants of lysyl oxidase eluted at the position shown above, and all five variants of TRAMP also co-eluted.

by the arrow in the elution profile (Fig. 3.9). The urea passed straight through the column leaving the protein to be eluted at its elution pH which is dependent on its pI (Fagerstam *et al.,* 1983). The pH at which each of the variants eluted is shown in Table 3.3.

The amino acid sequence of lysyl oxidase from various species (Trackman *et al.*, 1990; Trackman *et al.*, 1991; Hamalainen *et al.*, 1991; Wu *et al.*, 1992) is rich in acidic amino acids predicting a theoretical pI of approximately 6.3. By isoelectric focussing, lysyl oxidase variants are increasingly acidic with pI's in the pH range 5.80 +/- 0.2 to 5.20 +/- 0.2 (J.R.E. MacBeath, personal communication).

3.2.3 Amino acid analysis

Variants of lysyl oxidase were subjected to amino acid analysis (Table 3.4). No significant differences were found in the composition of the four variants. Their compositions are very similiar to that of the rat aorta (Trackman *et al.*, 1990; Trackman *et al.*, 1991), human placenta (Hamalainen *et al.*, 1991) and chick aorta (Wu *et al.*, 1992) enzymes derived from cDNA sequencing, given certain assumptions about the position of the pro-enzyme cleavage site (Table 3.5).

3.2.4 Mass analysis of the intact enzyme variants

The molecular masses of lysyl oxidase variants by SDS-PAGE were all approximately 32K (Fig. 3.7). More precise molecular mass determinations were obtained by mass spectrometry. Initially a Biolon 20 plasma desorption mass spectrometer was used for these analyses (Table 3.6), though this instrument was working towards the limit of its mass range. Later, a Lasermat laser desorption mass spectrometer instrument was used to determine the same molecular masses (Fig. 3.14a; Table 3.6). Each of the mass spectrometer instruments gave masses of approximately 29K with the Lasermat values being the more accurate. From the Lasermat data, the individual variants showed an 65Da incremental increase in mass in relation to their elution position from the Mono Q column (Fig. 3.10).

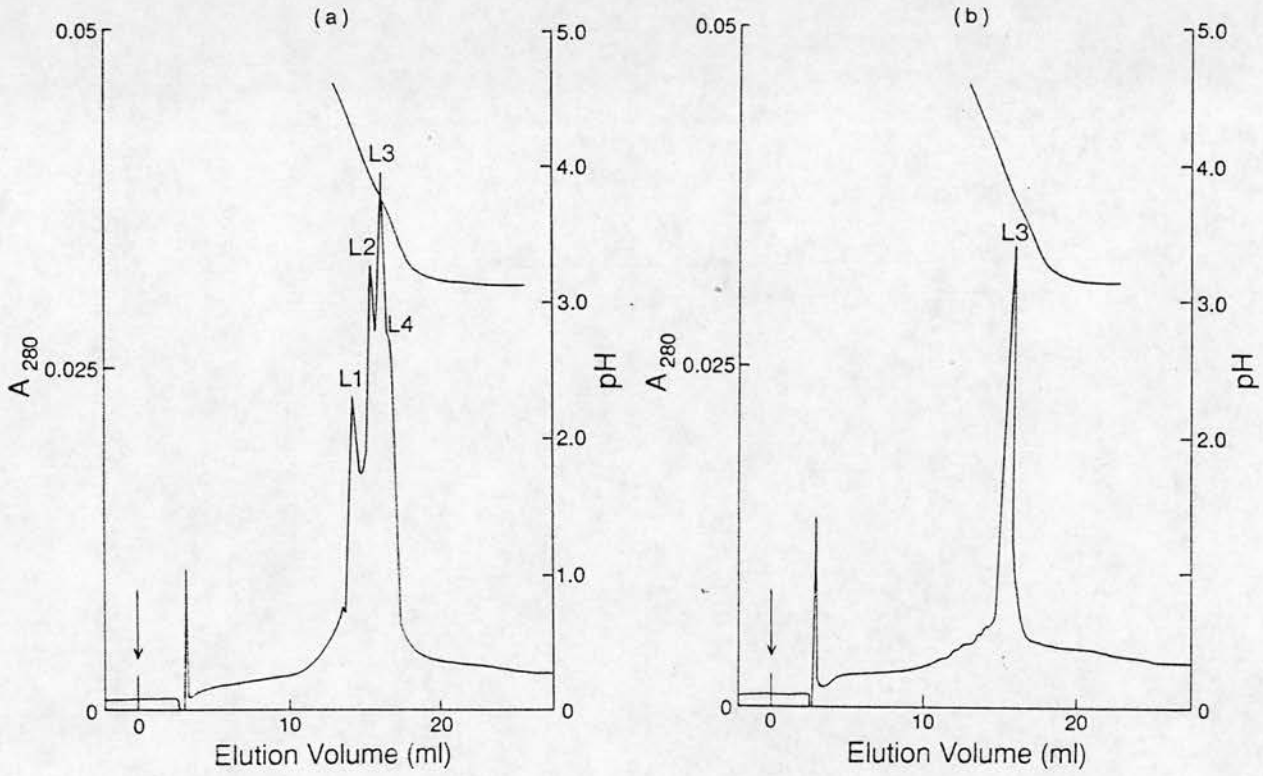


Fig. 3.9. Measurement of the elution pH of the different variants of lysyl oxidase by chromatofocussing chromatography

Samples were loaded onto a Mono P HR 5/20 chromatofocussing column (10 µm particle size; 5 mm x 200 mm) at a flow rate of 1 ml/min, previously equilibrated with 0.025 M Bis-Tris, pH 4.70 adjusted with HCl. Elution was with Pharmacia Polybuffer 74, diluted 1: 10 with water, pH 3.15, adjusted with HCl, from a 50 ml "superloop". The point of eluent application is shown by the arrow. A flow rate of 1 ml/min was used. The pH of each 0.5 ml fraction was measured using a pH meter. (a) Mixture of four types of lysyl oxidase (b) L3.

Table 3.3. Mono P chromatofocussing column elution pH of different variants of lysyl oxidase and TRAMP

Protein	Elution pH (24°C +/- 1.5°C)	pI
L1	4.38	5.80 +/- 0.2
L2	4.05	5.60 +/- 0.2
L3	3.72	5.40 +/- 0.2
L4	3.61	5.20 +/- 0.2
T1	ND	4.43 +/- 0.1
T2	3.42	4.34 +/- 0.06
T3	3.25	4.27 +/- 0.08
T4	3.06	4.18 +/- 0.08
T5	2.81	4.07 +/- 0.06

The pH values are believed to be accurate to +/- 0.1 pH unit.

The pI values were found by isoelectric focussing (data with permission of J.R.E. MacBeath).

Table 3.4. Amino acid composition of the four variants of lysyl oxidase

Amino acid	L 1	L 2	L 3	L 4	Human placenta[a]
D	26.6	29.0	28.7	29.2	33
E	16.8	17.3	18.0	18.6	17
S	21.4	20.6	22.2	22.4	18
G	14.4	14.4	14.0	14.6	15
H	11.2	12.4	12.7	10.9	11
R	17.9	18.6	19.1	17.1	18
T	12.0	11.6	11.6	12.3	14
A	16.0	16.0	16.0	16.0	15
P	21.6	22.1	21.6	21.3	16
Y	24.7	23.7	26.1	26.2	31
V	12.4	12.1	12.7	12.3	12
M	2.6	3.1	3.0	2.9	3
PEC	6.1	6.1	6.4	5.8	11
I	7.0	7.2	7.5	7.1	7
L	15.6	16.2	16.1	15.8	14
F	7.2	7.0	6.9	7.0	6
K	3.9	3.9	4.0	3.8	6
W	ND	ND	ND	ND	4
Totals	237.4	241.3	246.6	243.3	251

Values are residues per molecule, normalised on the assumption that the mature form of lysyl oxidase contains 16 alanine residues. ND = not determined. The values shown are the residues/molecule based on data from three time courses with hydrolysis times of 30, 60, and 90 min at 200°C. Tryptophan was not determined. The 420A amino acid analyser tends to overestimate G and underestimate E. The precision of these values are believed to be within 5% based on amino acid analysis of 500 pmol test peptide (ABI) used to ensure that the instrument was working within the manufacturer's specifiction.

[a] from cDNA sequencing of the human lysyl oxidase precursor (Hamalainen *et al.*, 1991), assuming the mature enzyme consists of the entire C-terminal region begining at residue 2, (Fig. 3.13.ii; Section 3.2.6). Note: D = 20, N = 13, E = 9, and Q = 8 in human placental lysyl oxidase cDNA derived data.

Table 3.5. Amino acid compositions of lysyl oxidase from various species

Amino acid	Porcine[a] skin	Rat[b] aorta	Human[c] placenta	Chick[d] aorta	Bovine[e] aorta	Chick[f] cartilage
D	28.7	32	33	34	25	33
E	18.0	19	17	17	27	26
S	22.2	23	18	23	20	20
G	14.0	13	15	14	21	24
H	12.7	12	11	10	6	7
R	19.1	18	18	18	12	14
T	11.6	12	14	12	12	13
A	16.0	15	15	14	16	16
P	21.6	16	16	16	12	14
Y	26.1	30	31	33	5	16
V	12.7	13	12	12	8	10
M	3.0	3	3	3	4	4
C	6.4	11	11	10	4	7
I	7.5	7	7	8	6	10
L	16.1	13	14	13	16	16
F	6.9	6	6	5	6	6
K	4.0	5	6	6	7	8
W	ND	3	4	3	ND	ND
Totals	246.6	251	251	251	207	244

ND = not determined.

[a] variant L3 (see Fig. 3.6a). Values are residues per molecule, normalised to 16 alanine residues.

[b] from cDNA sequencing of the rat lysyl oxidase precursor (Trackman *et al.*, 1990; Trackman *et al.*, 1991), assuming the mature enzyme consists of the entire C-terminal region begining at residue 2 (Fig. 3.13.ii).

[c] from cDNA sequence data of the human lysyl oxidase precursor (Hamalainen *et al.*, 1991), assuming the mature enzyme consists of the entire C-terminal region begining at residue 2 (Fig. 3.13.ii).

[d] from cDNA sequencing of the chick aorta lysyl oxidase precursor (Wu *et al.*, 1992), assuming the mature enzyme consists of the entire C-terminal region beginning at residue 3 (Fig. 3.13.ii).

[e] from data of Kagan *et al.* (1979) presented as an average of peaks I, II and III re-expressed as residues per molecule assuming 16 alanine residues.

[f] from data of Stassen (1976). This represents the composition of the fourth enzymically active peak eluted from DEAE-cellulose, re-expressed as residues per molecule assuming 16 alanine residues.

Table 3.6. Molecular masses of lysyl oxidase variants

Variant	Biolon 20[a]	Lasermat[b]
L1	29,496 +/- 300	29,239 +/- 30
L2	29,315 +/- 300	29,321 +/- 30
L3	29,351 +/- 300	29,377 +/- 30
L4	29,815 +/- 300	29,436 +/- 30

SDS-PAGE analysis showed that variants L1 to L4 had M_r ~ 32,000

[a] Biolon 20 values for proteins between 20,000 and 30,000 are accurate to +/-1% as the instrument is working at the limit of its range.

[b] Errors are standard deviations based on lysyl oxidase samples (n = 8), using carbonic anhydrase (M_r = 29,024) as an external standard.

The rat aorta LO sequence M_r = 29,015 (assumes start at D4 Fig. 3.13.ii).

The human LO sequence M_r = 29,107 (assumes start at D4 Fig. 3.13.ii).

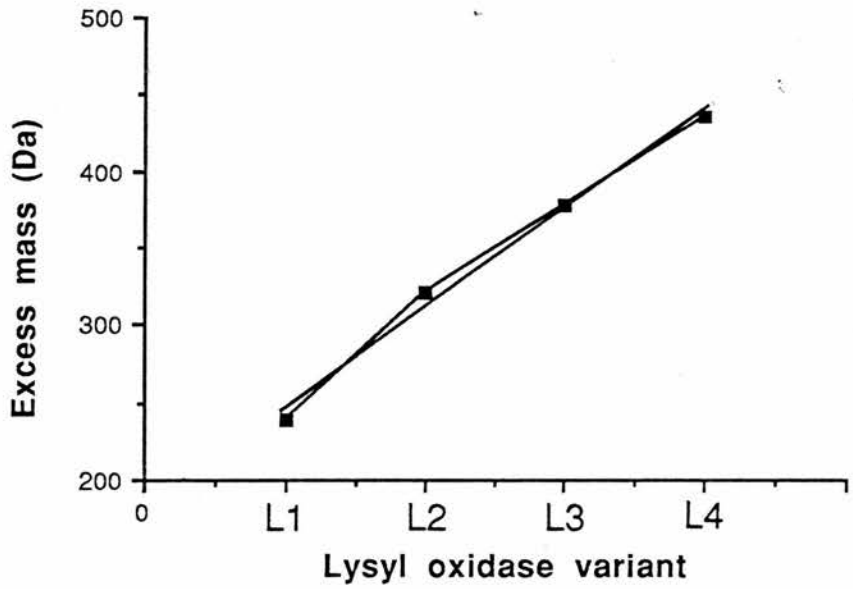


Fig. 3.10. Graph showing incremental mass changes between lysyl oxidase variants

The incremental mass difference between variants is 65Da.

3.2.5 Cyanogen bromide peptide analysis

Lysyl oxidase variant L3 was desalted using an Aquapore RP-300 C8 cartridge (Section 2.4.3; Fig. 3.8) and loaded onto the microsequencer. The enzyme was found to be N-terminally blocked and so it was necessary to chemically cleave and enzymically sub-digest the protein. The results are described below.

Variants of lysyl oxidase were treated with cyanogen bromide and the resulting unreduced peptides were separated by reverse phase HPLC (Fig. 3.11a-d). All the lysyl oxidase variants gave similar CNBr profiles. Four peptides were expected from inspection of the cDNA predicted sequence having made the assumption that mature lysyl oxidase had a M_r = 32K. However, only three peptides were found. The peaks in the L3 CNBr digest were sequenced and identified as the following peptides (1) DEF, (2) YNL and DEF, and (3) the blocked N-terminal peptide (Fig. 3.13; Tables 3.7 and 3.8), where each peptide is identified by the single letter code of the first three residues. Peptides YNL and DEF in peak 2 were attached by disulphide bonds. As DEF appeared as a single peak (peak 1) it may have become detached from YNL or have been sensitive to acid cleavage to the N-terminal side of a disulphide bond.

When L3 (unreduced) was treated with CNBr using an alternative method, whereby 20 times larger volumes of formic acid and the same CNBr concentrations were used, and the digest was eluted under the same HPLC conditions as described previously apart from the use of a higher flow rate (Fig. 3.11e), the first peak to elute was YNL and not DEF.

The peptides produced after digestion with CNBr were also analysed on a Lasermat mass analyser (Fig. 3.14b, c; Table 3.9). Values were found for the peptides YNL, DEF and the combined YNL + DEF peptide that was linked by disulphide bonds. Peak 3 from Fig. 3.11c gave no sequence. Confirmation that this was the N-terminal peptide came from mass analysis and sub-digestion of the peptide with endoproteinase-Asp-N. Mass analysis of the N-terminal peptide gave a value of M_r = 7,622 which was present with some contamination of peptide YNL, with M_r = 7,822 (Fig. 3.14c). The N-terminal mass value was found to be lower than expected from the cDNA sequence

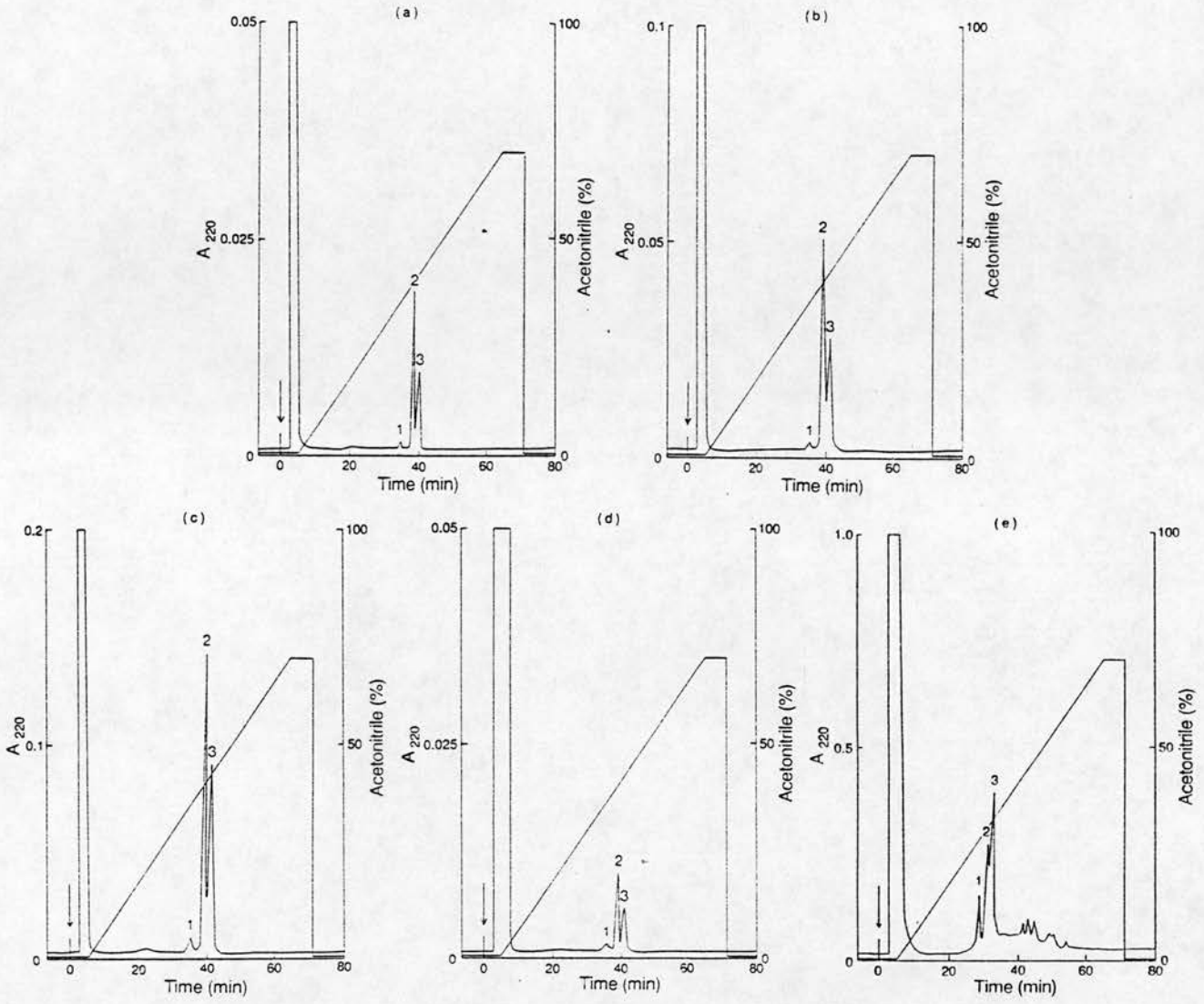


Fig. 3.11. Separation of peptides released by CNBr digestion of lysyl oxidase by reverse phase chromatography

Samples were separated on an Aquapore RP-300 C8 column (7μm particle size; 1 mm x 250 mm) at a flow rate of 100 μl/min. (a) CNBr digest of L1, non-reduced, (b) CNBr digest of L2, non-reduced, (c) CNBr digest of L3, non-reduced, (d) CNBr digest of L4, non-reduced. The peaks in (c) were later identified by sequence analysis and mass analysis using a Lasermat mass analyser as follows: (1) DEF, (2) YNL and DEF, (3) the blocked N-terminal peptide. (e) CNBr digest of L3, non-reduced, prepared by an alternative method (Section 3.2.5) and eluted under the same conditions as above but at a higher flow rate of 120 μl/min compared to 100 μl/min. The peaks were later identified by sequence analysis as follows: (1) YNL, (2) YNL and DEF, (3) the blocked N-termninal peptide. The significance of this separation is that YNL was eluted as peak 1 which contrasts with DEF as peak 1 in (c). The single letter code for the first three residues is used to define each peptide.

data from rat aorta (Trackman *et al.*, 1990; Trackman *et al.*, 1991), and human placental (Hamalainen *et al.*, 1991) lysyl oxidase, but consistent with the 29K laser desorption mass value of the entire enzyme variant L3 (Fig. 3.14a; Table 3.9) described in Section 3.2.4.

3.2.6 N-terminal sequence of variant L3

The L3 N-terminal CNBr peptide was sub-digested with endoproteinase-Asp-N which cleaves on the N-terminal side of aspartate, and sometimes, glutamate residues (Fig. 3.12). The endoproteinase-Asp-N peptides were identified by sequence analysis (Fig. 3.13; Tables 3.7 and 3.8) and mass analysis (Table 3.10) as follows (3) DDN, (4) DPY, (6) N-terminally blocked peptide believed to be DDP, (7) ERP, (8) endoproteinase-Asp-N, and (9) L3 CNBr peak 3, undigested by endoproteinase-Asp-N. Some peptides, e.g. DDN, eluted in more than one peak. They perhaps contained a post-translational modification. The tripeptides DTY and DLV were not found as they were probably too short to be retained by the reverse phase column.

Peak 6 gave a sequence beginning with DPY (initial yield = 10 pmol) which was sequenced through to completion. This compared to peak 4 where the initial yield was 40 pmol for peptide DPY. Peak 6 had a mass value of 1,272 Da (Table 3.10). After sequencing the minor peptide DPY present in peak 6, the glass fibre filter which still retained the blocked peptide was removed from the sequencer and exposed to 3M HCl vapour at 100°C for 15 min (Tsung and Frankel-Conrat, 1965) and then replaced in the sequencer for re-sequencing. A new sequence, but with a low initial yield of approximately 5 pmol, was observed which was believed to be the N-terminal sequence of the previously blocked peptide (Fig. 3.13; Tables 3.7 and 3.8). The mass value of the peak 6 peptide and the above sequence data suggest that the sequence of the major peptide and hence the N-terminus of the L3 variant of lysyl oxidase starts at aspartate residue D4 (Fig. 3.13.ii) and not, as predicted for the rat aorta lysyl oxidase, at residue R135 (= R170; Fig. 3.13.i; Trackman *et al.*, 1990; Trackman *et al.*, 1991) or for the human lysyl oxidase at residue R141 (= R170; Fig. 3.13.i; Hamalainen *et al.*, 1992; Trackman *et al.*, 1991).

A schematic diagram of the lysyl oxidase sequence based on mass

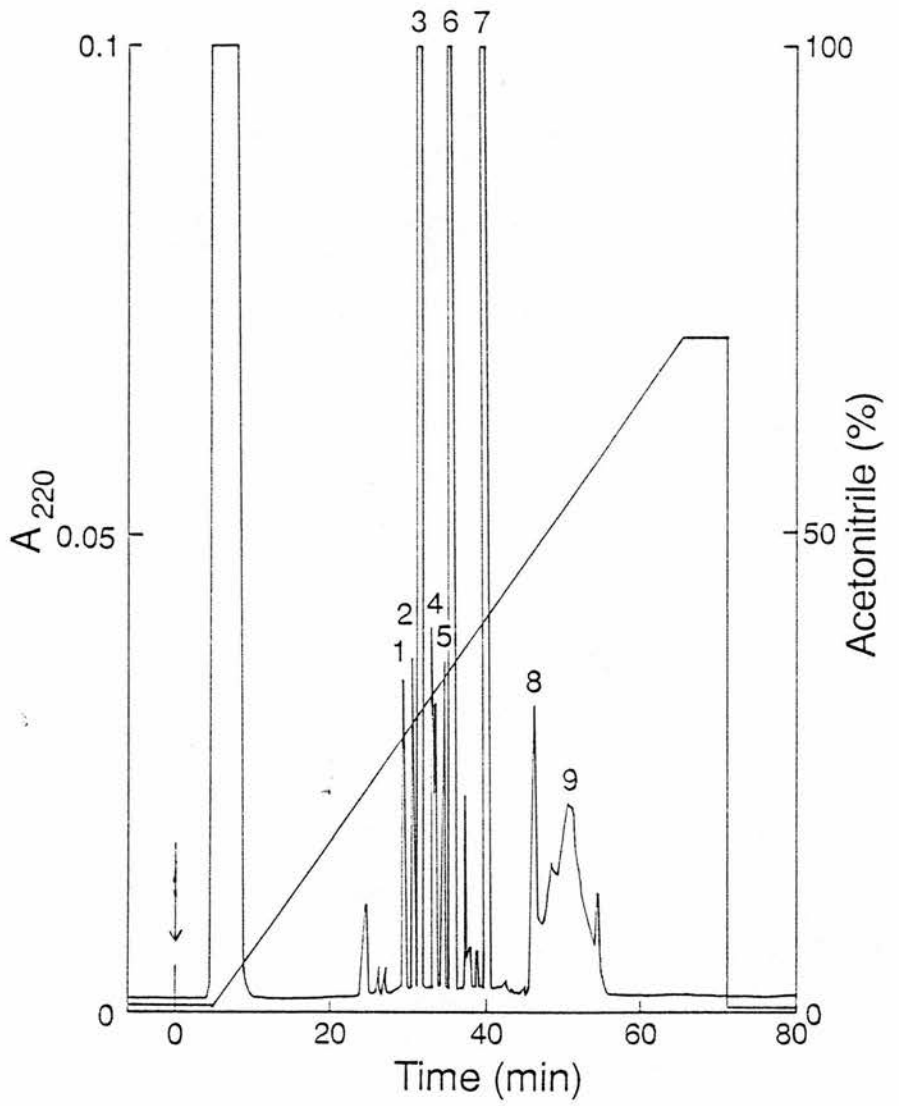


Fig. 3.12. Separationof peptides released by endoproteinase-Asp-N sub-digestion of L3 CNBr Fraction 3 by reverse phase chromatography

Samples were separated on an Aquapore RP-300 C8 cartridge (7μm particle size; 2.1 mm x 30 mm) at a flow rate of 100 μl/min.

The fractions were subsequently found to be as follows: (1) DDN [IY=17pmol], (2) DDN [IY = 28 pmol], (3) DDN [IY = 75 pmol], (4) DPY [IY = 40pmol], (5) DPY [IY = 19 pmol], (6) DPY [IY = 10 pmol] and N-terminally blocked peptide, DDP, (7) ERP [IY = 79 pmol], (8) endoproteinase-Asp-N, (9) L3 CNBr fraction 3 undigested by endoproteinase-Asp-N. IY = initial yield.

Fig. 3.13. Precursor region and mature form of lysyl oxidase amino acid sequence (predicted from cDNA) from various species

The figure includes data from chick aorta (Wu *et al.*, 1992), human placenta (Hamalainen *et al.*, 1991), rat aorta (Trackman *et al.*,1990 and 1991) lysyl oxidase and the murine *ras* recision gene (Mariani *et al.*, 1992) and amino acid sequence determined for porcine lysyl oxidase. The following symbols are used in the figure: - denotes identical amino acid residues; * denotes deletion in sequence; ? denotes unknown residue; + denotes sequence continues; rrg denotes the mouse *ras* recision gene. Amino acids in outlined type indicate a residue change in porcine lysyl oxidase compared to that of a known sequence. Bold letters are used for the first three resdues of each peptide sequenced.

i. Precursor region of lysyl oxidase

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Chick	M	R	C	A	P	P	G	L	L	L	A	Q	L	H	A	C	I	F	W	S
Human	-	-	F	-	W	T	V	-	L	-	G	P	-	Q	L	-	A	L	V	H
Rat	-	-	F	-	W	T	V	-	F	-	G	Q	-	Q	F	-	P	L	L	R
rrg	-	-	F	-	W	A	V	-	L	-	G	P	-	Q	L	-	P	L	L	R

	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Chick	G	L	W	P	A	*	G	C	Q	S	*	*	*	*	P	P	A	A	*	*
Human	C	A	P	P	-	A	G	Q	Q	Q	P	P	R	E	-	-	-	-	P	G
Rat	C	A	P	*	*	*	*	*	*	Q	A	P	R	E	-	-	-	-	P	G
rrg	C	A	P	*	*	*	*	*	*	Q	T	P	R	E	-	-	-	-	P	G

	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Chick	*	W	R	Q	R	I	Q	W	E	N	N	G	Q	V	Y	S	L	L	S	Q
Human	A	-	-	-	Q	-	-	-	-	-	-	-	Q	-	F	-	-	-	-	L
Rat	A	-	-	-	T	-	-	-	-	-	-	-	G	-	F	-	-	-	-	L
rrg	A	-	-	-	T	-	-	-	-	-	-	-	Q	-	F	-	-	-	-	L

	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
Chick	G	A	Q	Y	Q	P	P	R	R	R	Q	G	A	E	P	A	S	*	*	*
Human	-	S	-	-	-	-	Q	R	-	-	D	P	G	A	A	V	P	G	A	A
Rat	-	A	-	-	-	-	Q	Q	-	-	D	S	S	A	T	A	P	R	A	D
rrg	-	A	-	-	-	-	Q	R	-	-	D	P	S	A	T	A	R	R	P	D

	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
Chick	*	*	*	*	*	*	*	*	S	P	V	L	L	L	R	G	N	G	S	V
Human	N	A	S	A	Q	Q	P	R	T	-	I	-	-	I	-	D	-	R	T	A
Rat	G	N	A	A	A	Q	P	R	T	-	I	-	-	L	-	D	-	R	T	A
rrg	G	D	A	A	S	Q	P	R	T	-	I	-	-	L	-	D	-	R	T	A

	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Chick	P	R	A	A	A	*	*	*	*	*	*	A	A	A	A	R	P	Q	P	E
Human	A	A	R	T	R	T	A	G	S	S	G	V	T	-	G	-	-	R	-	T
Rat	S	A	R	A	R	T	P	S	P	S	G	V	A	-	G	-	-	R	-	A
rrg	S	T	R	A	R	T	P	S	P	S	G	V	A	-	G	-	-	R	-	A

	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
Chick	P	Q	P	Q	A	Q	P	Q	P	R	P	R	S	S	R	R	Q	P	L	G
Human	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Rat	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
rrg	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160
Chick	R	R	H	W	F	Q	A	G	Y	R	A	P	S	G	S	A	R	P	A	P
Human	*	-	-	-	-	-	A	-	-	*	*	*	*	*	*	*	*	*	S	T
Rat	*	-	-	-	-	-	V	-	F	*	*	*	*	*	*	*	*	*	S	P
rrg	*	-	-	-	-	-	A	-	F	*	*	*	*	*	*	*	*	*	S	P

Processing site predicted by

I Trackman *et al.*, (1991)

	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
Chick	R	R	R	P	R	G	R	R	S	R	R	R	E	R	A	E	R	R	R	A
Human	S	R	A	R	E	R	G	A	S	-	A	E	N	Q	T	A	P	G	E	V
Rat	S	G	A	G	D	G	A	S	R	-	A	A	N	R	T	A	S	P	Q	P
rrg	S	G	A	R	D	G	A	S	R	-	A	A	N	R	T	A	S	P	Q	P

	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195
Chick	A	A	P	S	G	L	R	P	G	*	R	E	D	V	M
Human	P	A	L	-	N	-	-	-	P	S	R	V	-	G	-
Rat	P	Q	L	-	N	-	-	-	P	S	H	V	-	R	-
rrg	P	Q	L	-	N	-	-	-	P	S	H	I	-	R	-

ii. Mature form of lysyl oxidase

The numbering is completely arbitrary and begins at M195 (above) which has been renumbered below as M1. The CNBr peptide beginning VGD was not found by sequence or mass analysis. The endoproteinase-Asp-N peptide beginning DDP has been tentatively identified but the sequencing yields were low (Section 3.2.6). The N-terminus of porcine lysyl oxidase may start at the aspartate residue D4.

				I																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Chick	M	V	G	D	D	P	Y	S	P	Y	K	Y	T	D	D	N	P	Y	Y	N
Human	-	-	-	-	-	-	-	N	-	-	-	-	S	-	-	-	-	-	-	-
Rat	-	-	-	-	-	-	-	N	-	-	-	-	S	-	-	-	-	-	-	-
rrg	-	-	-	-	-	-	-	N	-	-	-	-	S	-	-	-	-	-	-	-
Pig CNBr																				
CNBr (red)																				
Asp-N				**D**	**D**	**P**	Y	N	P	Y	?	Y	?	**D**	**D**	**N**	P	Y	Y	N

	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Chick	Y	Y	D	T	Y	E	R	P	R	Q	G	S	R	Y	R	P	G	Y	G	T
Human	-	-	-	-	-	-	-	-	-	P	-	G	-	Y	-	-	-	-	-	-
Rat	-	-	-	-	-	-	-	-	-	S	-	S	-	H	-	-	-	-	-	-
rrg	-	-	-	-	-	-	-	-	-	P	-	S	-	N	-	-	-	-	-	-
Pig CNBr																				
CNBr (red)																				
Asp-N	Y	Y				**E**	**R**	**P**	R	P	G	S	R	Y	R	P	G	Y	G	T

	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Chick	G	Y	F	Q	Y	G	L	P	D	L	V	P	D	P	Y	Y	I	Q	A	S
Human	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-
Rat	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-
rrg	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-
Pig CNBr																				
CNBr (red)																				
Asp-N	G	Y	F	Q	Y	G	L	P					**D**	**P**	**Y**	Y	I	Q	A	S

	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
Chick	T	Y	V	Q	R	**M**	S	**M**	Y	N	L	R	C	A	A	E	E	N	C	L
Human	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rrg	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pig CNBr									**Y**	**N**	**L**	R	C	A	A	E	E	N	C	L
CNBr (red)									**Y**	**N**	**L**	R	C	A	A	E	E	N	C	L
Asp-N	T	Y	V	Q	K															

	81	82	83	84	85	86	87	88	89	90	91	92	93	94	959	96	97	98	99	100
Chick	A	S	S	A	Y	R	A	D	V	R	D	Y	D	N	R	V	L	L	R	F
Human	-	-	T	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-
Rat	-	-	S	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-
rrg	-	-	S	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-
Pig CNBr	A	S	T	A	Y	R	A	D	V	R	D	Y	D	H	R	V	L	L	R	F
CNBr (red)	A	S	T	A	Y	R	A	D	V	R	D	Y	D	H	R	V	L	L	R	F

	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Chick	P	Q	R	V	K	N	Q	G	T	S	D	F	L	P	S	R	P	R	Y	S
Human	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rrg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pig CNBr	P	Q	R	V	K	N	Q	G	T	S	D	F	L	P	S	R	P	R	Y	S
CNBr (red)	P	Q	R	V	K	N	?	G	+											

	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
Chick	W	E	W	H	S	C	H	Q	H	Y	H	S	**M**	D	E	F	S	H	Y	D
Human	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	Y
Rat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	D
rrg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	D
Pig CNBr	?	E	?	S	+									**D**	**E**	**F**	S	H	Y	D
CNBr (red)														**D**	**E**	**F**	S	H	Y	D

	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160
Chick	L	L	D	A	S	S	H	R	K	V	A	E	G	H	K	A	S	F	C	L
Human	-	-	-	-	N	T	Q	-	R	W	-	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-	S	T	Q	-	R	V	-	-	-	-	-	-	-	-	-	-
rrg	-	-	-	-	N	T	Q	-	R	V	-	-	-	-	-	-	-	-	-	-
Pig CNBr	L	L	D	A	S	T	Q	R	R	V	A	E	G	-	-	A	S	F	C	L
CNBr (red)	L	L	D	A	S	T	Q	R	R	V	A	E	G	H	K	A	S	F	C	L

	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
Chick	E	D	T	S	C	D	Y	G	Y	Y	R	R	Y	A	C	T	A	H	T	Q
Human	-	-	-	-	-	-	-	-	-	H	-	-	F	-	-	-	-	-	-	-
Rat	-	-	-	-	-	-	-	-	-	H	-	-	F	-	-	-	-	-	-	-
rrg	-	-	-	-	-	-	-	-	-	H	-	-	F	-	-	-	-	-	-	-
Pig CNBr	E	D	T	S	C	D	Y	G	Y	?	R	R	F	A	C	T	A	?	T	Q
CNBr (red)	E	+																		

	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200
Chick	G	L	S	P	G	C	Y	D	T	Y	N	A	D	I	D	C	Q	W	I	D
Human	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-
rrg	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-
Pig CNBr	G	L	S	P	G	?	Y	+												
CNBr (red)																				

	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220
Chick	I	T	D	V	K	P	G	N	Y	I	L	K	V	S	V	N	P	S	Y	L
Human	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rrg	-	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240
Chick	V	P	E	S	D	Y	S	N	N	I	V	R	C	D	I	R	Y	T	G	H
Human	-	-	-	-	-	-	T	-	-	V	-	-	-	D	-	-	-	-	-	-
Rat	-	-	-	-	-	-	S	*	-	V	-	-	-	E	-	-	-	-	-	-
rrg	-	-	-	-	-	-	T	-	-	V	-	-	-	D	-	-	-	-	-	-

	241	242	243	244	245	246	247	248	249	250	251	252
Chick	H	A	Y	A	S	G	C	T	I	S	P	Y
Human	-	-	-	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-	-	-	-	-	-	-	-	-
rrg	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.7. Yields of amino acid residues determined from peptide sequencing of L3

The columns show yields, in pmol, from sequencing different peptides. The columns show (from left to right): lysyl oxidase cleaved by CNBr unreduced (CNBr) and reduced (CNBr(red)), and by endoproteinase-Asp-N (D). The numbering scheme is as in Fig. 3.13.ii.

Position	Residue	CNBr	CNBr(red)	D
1	M			
2	V			
3	G			
4	D			5 **DDP**
5	D			4
6	P			9
7	Y			2
8	N			3
9	P			7
10	Y			1
11	K			?
12	Y			1
13	S			?
14	D			41 **DDN**
15	D			46
16	N			44
17	P			95
18	Y			61
19	Y			61
20	N			48
21	Y			51
22	Y			39
23	D			
24	T			
25	Y			**ERP**
26	E			36
27	R			9
28	P			59
29	R			9
30	P			61
31	G			37
32	S			12
33	R			9
34	Y			25
35	R			4
36	P			36
37	G			28
38	Y			22
39	G			22
40	T			8
41	G			18
42	Y			12
43	F			3
44	Q			2
45	Y			9
46	G			8

Position	Residue	CNBr	CNBr(red)	D
47	L			4
48	P			1
49	D			
50	L			
51	V			
52	P			**DPY**
53	D			34
54	P			19
55	Y			38
56	Y			38
57	I			40
58	Q			29
59	A			32
60	S			14
61	T			11
62	Y			18
63	V			17
64	Q			14
65	K			4
66	M			
67	S			
68	M	**YNL**	**YNL**	
69	Y	182	72	
70	N	182	67	
71	L	212	84	
72	R	104	18	
73	C	128	43	
74	A	134	70	
75	A	(Low cycle)80	74	
76	E	106	50	
77	E	104	45	
78	N	110	40	
79	C	148	28	
80	L	112	44	
81	A	149	43	
82	S	52	19	
83	**T**	62	20	
84	A	98	36	
85	Y	78	26	
86	R	62	15	
87	A	112	28	
88	D	67	19	
89	V	75	18	
90	R	51	8	
91	D	65	20	
92	Y	69	17	
93	D	44	19	
94	H	?	4	
95	R	43	13	
96	V	57	22	
97	L	49	15	
98	L	65	20	
99	R	42	14	
100	F	31	18	
101	P	58	17	
102	Q	32	10	

D

Position	Residue	CNBr	CNBr(red)
103	R	37	19
104	V	41	12
105	K	23	2
106	N	26	6
107	Q	24	?
108	G	33	13
109	T	15	+
110	S	12	
111	D	?	(Poor injection)
112	F	12	
113	L	16	
114	P	29	
115	S	11	
116	R	18	
117	P	25	
118	R	18	
119	Y	26	
120	S	8	
121	W	?	
122	E	6	
123	W	?	
124	H	?	
125	S	8	
126	C	+	
127	H		
128	Q		
129	H		
130	Y		
131	H		
132	S		
133	M	**DEF**	**DEF**
134	D	108	124
135	E	171	13
136	F	244	100
137	S	90	48
138	H	?	14
139	Y	94	19
140	D	(Low cycle)37	80
141	L	147	73
142	L	174	103
143	D	149	83
144	A	151	64
145	S	57	27
146	T	70	29
147	Q	77	35
148	R	68	61
149	R	81	70
150	V	83	47
151	A	90	39
152	E	44	24
153	G	65	35
154	H	?	7
155	K	?	5
156	A	60	26
157	S	22	10
158	F	24	18

Position	Residue	CNBr	CNBr(red)	D
159	C	44	1	
160	L	37	14	
161	E	20	11	
162	D	36	+	
163	T	6		
164	S	17		
165	C	27		
166	D	31		
167	Y	43		
168	G	32		
169	Y	44		
170	H	?		
171	R	25		
172	R	28		
173	F	12		
174	A	23		
175	C	19		
176	T	3		
177	A	23		
178	H	?		
179	T	11		
180	Q	11		
181	G	20		
182	L	11		
183	S	8		
184	P	23		
185	G	17		
186	C	?		
187	Y	25		
188	D	+		
189	T			
190	Y			

The following symbois are used in the above table: ? denotes residue unknown; + denotes sequence continues.

Table 3.8. Repetitive yields of lysyl oxidase peptides sequenced

i.	Unreduced CNBr peptides	Repetitive yield (%)	Residues used
	YNL	93	
	DEF	93	N,E,L
ii.	Reduced CNBr peptides		
	YNL	92	Y,N,A,V,Q
	DEF	91	F,S,H
iii.	Endoproteinase-Asp-N peptides		
	DDP	92	P
	DDN	94	Y
	ERP	94	Y
	DPY	88	Y,Q

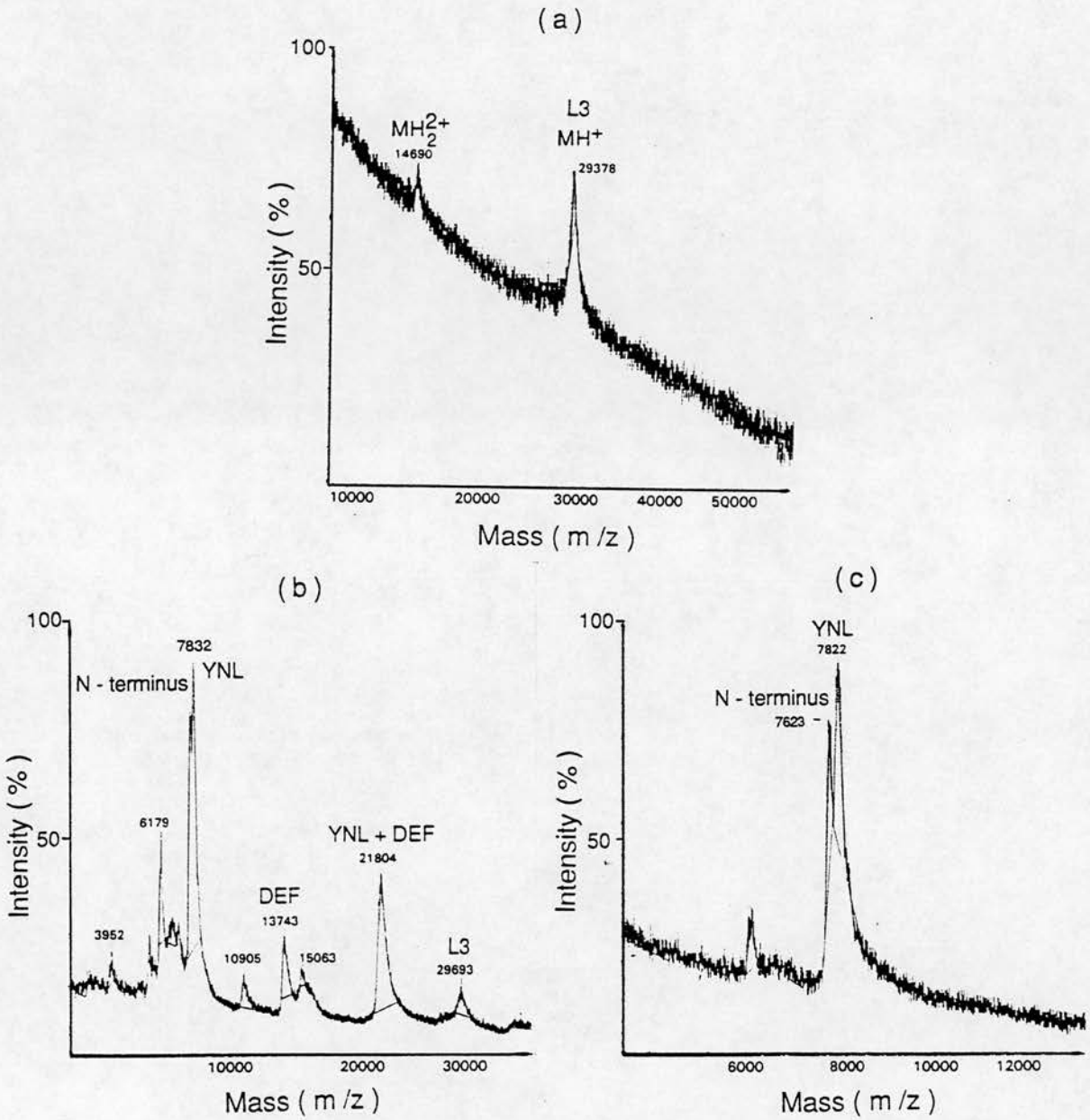


Fig. 3.14. Spectra from a Finnigan MAT Lasermat laser desorption mass analyser of L3 and L3CNBr peptides

(a) Approximately 100 pmol L3, M_r = 29,377. (b) Approximately 1 pmol L3CNBr digest. Interpretation shows L3 with M_r = 29,692 (29,377), peptides YNL + DEF, non-reduced, M_r = 21,803 (21,283*), peptide DEF M_r = 13,742 (13,464*), and unresolved peptides, YNL + the N-terminal peptide, M_r =7,831. (c) Approximately 1 pmol L3CNBr peak 3 (Fig. 3.11c). Interpretation shows peptide YNL M_r = 7,821 (7,838*) and the N-terminal peptide M_r = 7,622 (7,533*). The MH^+ ion gives the strongest signal with this instrument. Theoretical values are shown in parentheses. * assumes identity with human sequence.

Table 3.9. Mass analysis using a Lasermat mass analyser of peptides of L3 after cleavage with CNBr

Peptide	Theoretical M_r [a]	Theoretical M_r [b]	Lasermat M_r
N-terminal [c]	7,533.1	7,533.1	7,622 +/-1
Shs	206.2	206.2	ND
YNL	7,823.6	7,837.6	7,821 +/-1
DEF	13,365.5	13,463.7	13,742 +/-2
Total [d]	29,015	29,107	
Combination peptide			
YNL + DEF	21,171.1	21,283.3	21,803 +/-3

[a] assumes identity with rat sequence (Trackman *et al.*, 1990; Trackman *et al.*, 1991).

[b] assumes identity with human sequence (Hamalainen *et al.*, 1991).

[c] assumes N-terminal peptide starts with DDP (D4; Fig. 3.13.ii).

[d] from cDNA data

The following abbreviations are used in the above figure: hs = homoserine (Shs = dipeptide, Ser-homoserine).

ND = not determined, since the masses were too low to be detected on the Lasermat mass analyser.

Table 3.10. Mass analysis using a Lasermat mass analyser of L3 N-terminal CNBr fragment after sub-digestion with endoproteinase-Asp-N

Fraction	Peptide	Theoretical M_r [a]	Theoretical M_r [b]	Lasermat M_r
6	DDP	1,261.3	1,261.3	1,272 +/-1
3	DDN	1,226.2	1,226.2	1,245 +/-1
	DTY	397.4	397.4	ND
7	ERP	2,638.9	2,675.0	2,675 +/-1
	DLV	442.5	416.5	ND
4	DPY	1,676.8	1,676.8	1,678 +/-1
Total		7,553.1	7,533.1	7,622 +/-1
	Combination peptides			
1-7	DTY + ERP	3,018.3	3,054.4	3,055 +/-1
4	DLV + DPY	2,101.3	2,075.3	2,172 +/-1
4	DPY + MS	1,865.0	1865.0	1,868 +/-1

The peptides relate to fractions 1-7 in Fig. 3.12, and are identified in Table 3.7.

[a] assumes identity with the rat sequence (Trackman *et al.*, 1990; Trackman *et al.*, 1991).

[b] assumes identity with the human sequence (Hamalainen *et al.* 1991).

ND = not determined, since the masses were too low to be detected on the Lasermat mass analyser.

data of CNBr fragments is shown in Fig. 3.15. A peptide with M_r = 2,303 might be expected to be present if the human lysyl oxidase began at residue R141. A peptide of this mass was not found.

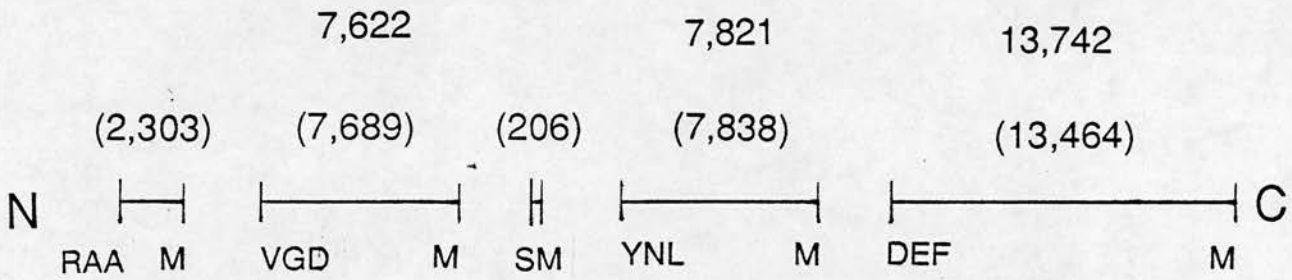
3.3 Tyrosine Rich Acidic Matrix Protein (TRAMP)

3.3.1 Separation of TRAMP variants

TRAMP, following purification by Sephacryl S-400 chromatography, (Pool 2; Fig. 3.4), was further purified by anion exchange Mono Q FPLC (Fig. 3.6b). Five variants of TRAMP (T1 to T5) were resolved. Peaks from the Mono Q column were analysed by SDS-PAGE (Fig. 3.16), and the electrophoretic migration of the five TRAMP variants was found to be identical. Similarly, all five variants of TRAMP eluted at the same position by reverse phase HPLC (Fig. 3.8). TRAMP was assayed for lysyl oxidase activity using [^{3}H]elastin as substrate (Section 2.1) and found to have no lysyl oxidase activity.

3.3.2 Chromatofocussing

A Mono P chromatofocussing column was equilibrated with buffer A (0.025M Bis-Tris, pH 3.65). The TRAMP sample in 6M PBU was applied to the column. When the pH was steady, buffer B (Pharmacia Polybuffer 74, pH 2.60) was applied to the column, as shown in Fig. 3.17. The urea passed straight through the column leaving the protein to be eluted at its elution pH which is dependent on its pI (Fagerstam *et al.,* 1983). The pH at which each of the variants eluted is shown in Table 3.3. The amino acid sequence of the TRAMP (Fig. 3.23) is rich in acidic amino acids. From complete sequence data for porcine TRAMP (Section 3.3.4; Fig. 3.23) the calculated isoelectric point (pI; determined with the University of Wisconsin GCG software package, and allowing for pyroglutamate at the N-terminus) is 4.53 (D.J.S. Hulmes, personal communication). To determine the pI experimentally, TRAMP variants were analysed (by J.R.E. MacBeath) by isoelectric focussing in the presence of 4M urea. The pI values for each variant are also shown in Table



141	167	residue numbers from Trackman *et al.*, (1991)
170	176 (or 2)	residue numbers from Fig. 3.13.i and 3.13.ii.

Fig. 3.15. Schematic diagram of lysyl oxidase sequence

The diagram shows observed mass values of CNBr peptides from porcine skin lysyl oxidase variant L3 (Fig. 3.14b, c). Shown beneath them, in parentheses, are the masses of expected CNBr fragments of human lysyl oxidase with adjustment where the porcine sequence is known to differ (Hamalainen *et al.*, 1991). The peptide resulting from a putative precursor processing site at arginine 141 (R141; = R170, Fig. 3.13; Trackman *et al.*, 1991) was not observed.

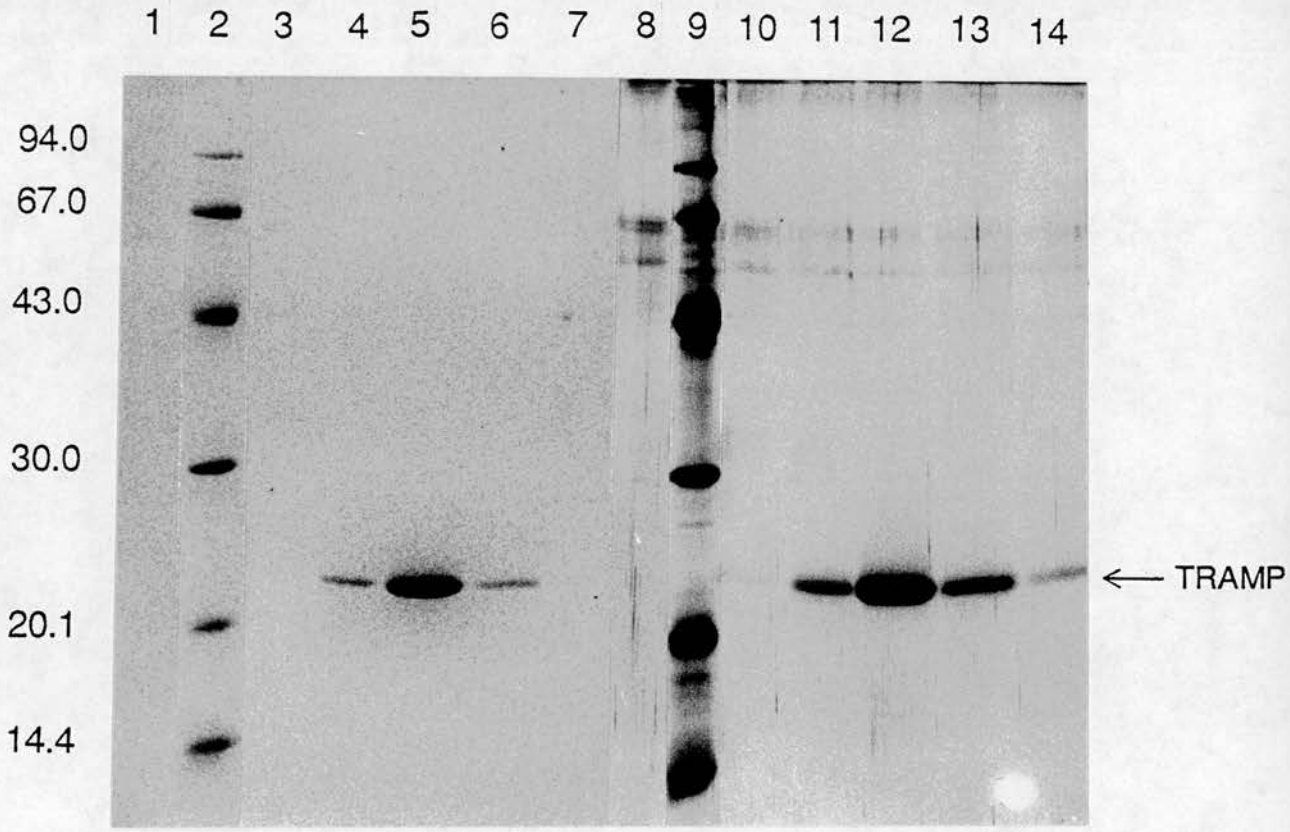


Fig. 3.16. SDS-PAGE analysis of the five variants of TRAMP separated by anion exchange chromatography

The lanes are: (1) blank, (2) protein standards, (3) T1, (4) T2, (5) T3, (6) T4, (7) T5, (8) blank, (9) protein standards, (10) T1, (11) T2, (12) T3, (13) T4, (14) T5. Lanes (1-7) were stained with Coomassie Blue, whilst lanes (8-14) were stained with silver stain. Approximately 1μg of protein was loaded in each lane and analysed (in reducing conditions) on a 12% SDS-PAGE gel. The molecular masses (K) of protein standards are indicated.

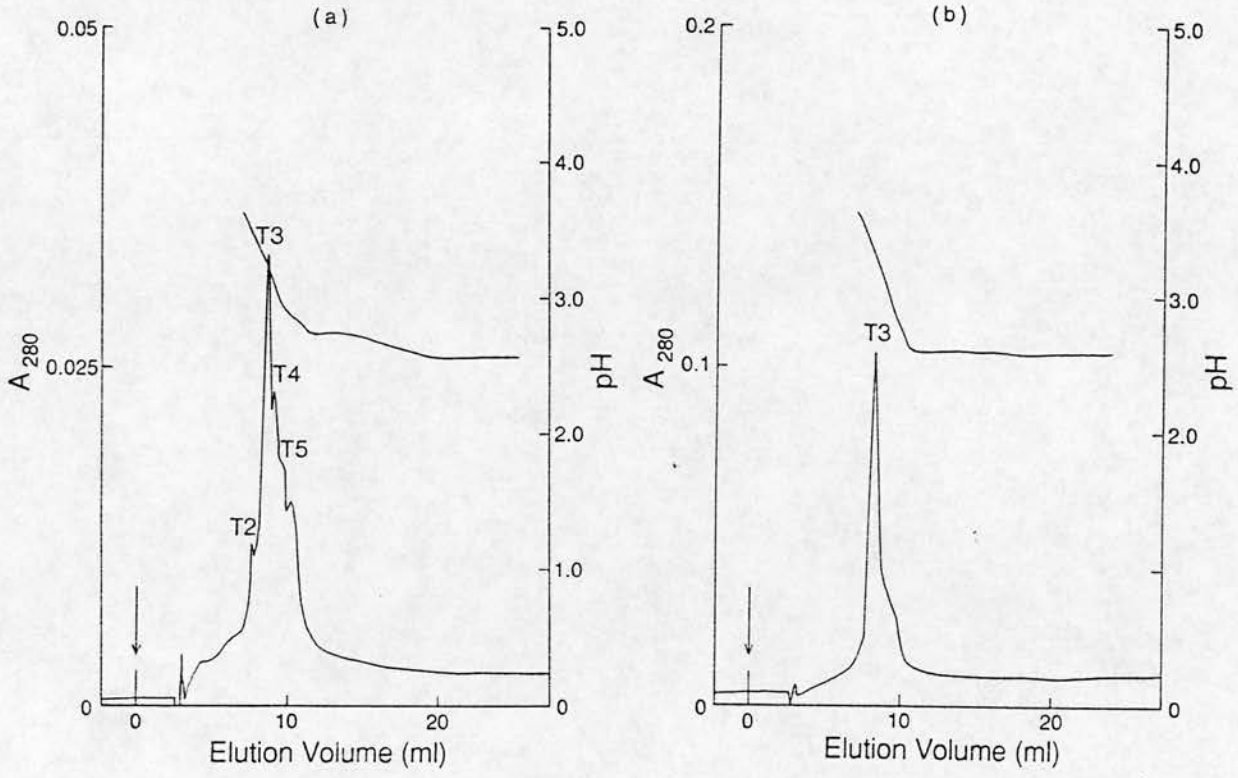


Fig. 3.17. Measurement of the elution pH of the different variants of TRAMP by chromatofocussing chromatography

Samples were loaded onto a Mono P HR 5/20 chromatofocussing column (10 µm particle size; 5mm x200mm), previously equilibrated with 0.025 M Bis-Tris, pH 3.65 adjusted with HCl at a flow rate of 1 ml/min. Elution was with Pharmacia Polybuffer 74, diluted 1: 10 with water (pH adjusted to 2.60 with HCl), from a 50 ml "superloop". The point of eluent application is shown by the arrow. (a) Mixture of the five variants of TRAMP, (b) T3.

3.3. TRAMP variants T1 to T5 are increasingly acidic with pI's in the range 4.43 to 4.07. The observed pI's are less than the theoretical value, and they decrease as expected from the Mono Q profile. The elution pH values from the Mono P chromatofocussing column are consistent with these data. The elution pH values are lower because once eluting pH reaches pI of the protein, the protein has to elute through a 200 mm column by which time the surrounding pH has dropped. The elution pH and pI values for the TRAMP variants were found to be more acidic than the corresponding values for the lysyl oxidase variants.

3.3.3 Amino acid analysis

Variants T1 to T5 of porcine skin TRAMP were subjected to amino acid analysis (Table 3.11). The most striking observation is the relative abundance of tyrosine, which accounts for about 10% of the molecule. This compares with an average protein composition which would normally contain about 3% tyrosine (Croft, 1976). No significant differences were found in the composition of the five variants. Amino acid compositions of the 23K contaminant found in lysyl oxidase preparations from human placenta (Kuivaniemi *et al.*, 1984) and of the dermatan sulphate proteoglycan-associated 22K protein from bovine skin (Neame *et al.*, 1989) are shown in Table 3.12. The composition of TRAMP is essentially identical to that of the bovine skin protein, and there are some similarities with the human placental 23K protein (Kuivaniemi *et al.*, 1984). A comparison of the amino acid analysis of the T3 variant of TRAMP with rat aorta lysyl oxidase (Trackman *et al.*, 1990; Trackman *et al.*, 1991) demonstrates how earlier workers believed TRAMP was a breakdown product of lysyl oxidase (Table 3.13).

3.3.4 Sequence analysis of T3 variant of TRAMP

TRAMP variant T3 was desalted using an Aquapore RP-300 C8 column (Section 2.4.3; Fig. 3.8) and loaded onto the microsequencer. The protein was found to be N-terminally blocked and so it was necessary to chemically cleave and enzymically sub-digest the protein. The results are

Table 3.11. Amino acid composition of the five variants of TRAMP

	Porcine skin						Bovine Skin Protein[b]	
	Amino acid analysis (AAA)					Sequence[a]	AAA	Sequence
	T1	T2	T3	T4	T5	T3		
D	18.6	19.7	19.6	20.4	19.6	20	20.1	20
E	22.8	23.5	23.6	23.9	24.1	25	22.9	24
S	12.1	12.4	11.9	11.7	12.5	13	12.6	13
G	12.6	12.9	13.1	12.9	13.0	13	14.6	13
H	2.8	3.0	2.9	3.0	2.9	3	3.8	4
R	11.7	12.3	12.3	12.8	12.5	12	12.8	12
T	7.3	7.0	7.4	6.7	6.5	8	7.3	9
A	7.0	7.0	7.0	7.0	7.0	7	7.1	7
P	6.6	6.5	6.7	6.6	6.5	7	9.7	6
Y	15.4	17.9	18.1	19.0	17.9	20	18.8	20
V	7.8	8.1	8.0	8.0	8.1	9	7.5	9
M	6.0	7.6	6.8	7.8	7.1	8	6.0	7
PEC	8.9	8.8	9.2	9.1	9.1	10	7.9	10
I	3.9	3.9	3.8	3.9	3.8	4	4.2	4
L	4.1	4.1	3.7	3.8	3.8	4	5.6	5
F	7.6	7.8	7.7	8.0	7.7	8	8.0	8
K	3.5	4.0	4.0	4.3	4.0	4	4.2	4
W	ND	ND	ND	ND	ND	8	ND	8

[a] Note: D = 11, N = 9, E = 14 and Q = 11.

[b] D = 11, N = 9, E = 14 and Q = 10. Data of Neame *et al.* (1989).

The values shown are the residues/molecule based on data from three time courses with hydrolysis times of 30, 60, and 90 min at 200°C. Tryptophan was not determined. The values were normalised on the assumption that there were 7 alanine residues/molecule as found in the bovine skin 22K protein (Neame *et al.*, 1989) and also in the porcine sequence (Fig. 3.23). The precision of these values are believed to be within 5% based on amino acid analysis of 500 pmol test peptide (ABI) used to ensure that the instrument was working within the manufacturer's specifiction.

Table 3.12. Amino acid composition of TRAMP and related proteins

Amino acid	Porcine skin [a]	Bovine skin [b] (22K protein)	Human placenta [c] (Contaminant, pool II)
D	19.6	20	14.1
E	23.6	24	21.9
S	11.9	13	10.5
G	13.1	13	14.3
H	2.9	4	2.5
R	12.3	12	9.0
T	7.4	9	6.8
A	7.0	7	7.0
P	6.7	6	5.9
Y	18.1	20	8.4
V	8.0	9	6.4
M	6.8	7	ND
C	9.2	10	ND
I	3.8	4	4.1
L	3.7	5	4.1
F	7.7	8	5.4
K	4.0	4	4.8
W	ND	8	ND

ND = not determined.

[a] variant T3 (see Fig. 3.6b). Values are residues per molecule, normalised to 7 alanine residues.

[b] from protein sequence data of Neame *et al.* (1989), expressed as residues per molecule.

[c] from Kuivamiemi *et al.* (1984) presented as the contaminant peak in pool II eluted from DEAE-cellulose, re-expressed as residues per molecule, assuming 7 alanine residues.

Table 3.13. Comparison of the sequence derived amino acid composition of T3 with rat aorta lysyl oxidase cDNA derived data

AA	Lysyl oxidase[a]	T3
D	22	11
N	10	9
E	9	14
Q	10	11
S	23	13
G	13	13
H	12	3
R	18	12
T	12	8
A	15	7
P	16	7
Y	30	20
V	13	9
M	3	8
C	11	10
I	7	4
L	13	4
F	6	8
K	5	4
W	3	8

[a] Data of Trackman *et al.* 1990 and Trackman *et al.* 1991 from rat aorta cDNA sequence, assuming the mature protein starts at residue 2 (Fig. 3.13.ii).

The table demonstrates how earlier workers believed that TRAMP, as it is now called, was a breakdown product of lysyl oxidase. Only glutamate, glutamine, methionine, phenylalanine, and tryptophan are more abundant in TRAMP than lysyl oxidase, with glutamine, methionine, and tryptophan being difficult to quantify by amino acid analysis.

described below.

3.3.4.1 Pyroglutamate aminopeptidase digestion of the T3 variant of TRAMP

The blocked N-terminus may have been due to the presence of a pyroglutamate group. The protein was therefore treated with pyroglutamate aminopeptidase. The digested protein was desalted by reverse phase HPLC (Fig. 3.18). This procedure unblocked the protein yielding the sequence beginning with YGD (Fig. 3.23; Tables 3.14 and 3.15).

3.3.4.2 Chemical removal of the blocking group of T3

T3 was also treated with 17% methylamine at 37°C for 14h, desalted by reverse phase HPLC and subjected to amino acid sequencing (Muranova and Muranov, 1979). This failed to unblock the protein, possibly because the pyroglutamate was adjacent to a tyrosine group, which reduces the reactivity of the pyrrolidone ring (Muranova and Muranov, 1979).

T3 was also dried onto a glass fibre filter and treated with 3M HCl vapour at 100°C for 15 min and subjected to sequencing (Tsung and Frankel-Conrat, 1965). This failed to give any sequence data. However, when the first procedure with 17% methylamine was followed by exposure to 3M HCl vapour at 100°C for 15 min, the N-terminal sequence of T3 was detected (Table 3.15). The aminopeptidase treatment was the preferred method but a chemical procedure would have been simpler if it had been more reliable.

3.3.4.3 Cyanogen bromide digestion of TRAMP

The T2 to T5 variants of TRAMP were treated with cyanogen bromide and the resulting unreduced peptides were separated by reverse phase HPLC (Fig. 3.19a-d). All the TRAMP variants gave similiar CNBr profiles, which were quite distinct from the equivalent lysyl oxidase CNBr profiles (Fig. 3.11). Eight peptides were expected. However, the dipeptide, CR was too small to be detected by reverse phase chromatography. The peaks in the T3

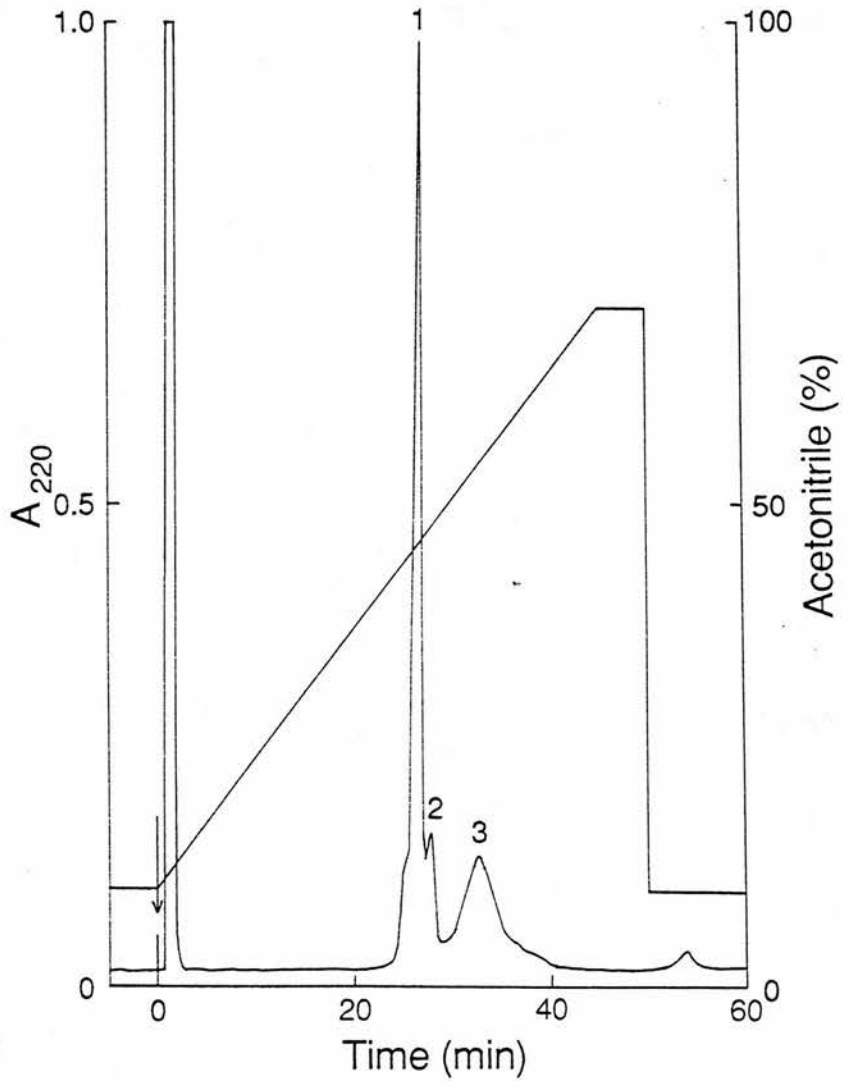


Fig. 3.18. Separation of T3 after pyroglutamate aminopeptidase digestion by reverse phase chromatography

The sample was desalted on an Aquapore RP-300 C8 cartridge (7μm particle size; 2.1 mm x 30 mm) at a flow rate of 100 μl/min. The peaks were identified as follows: (1) unblocked T3, (2) pyroglutamate aminopeptidase and (3) glycerol from the reaction mixture.

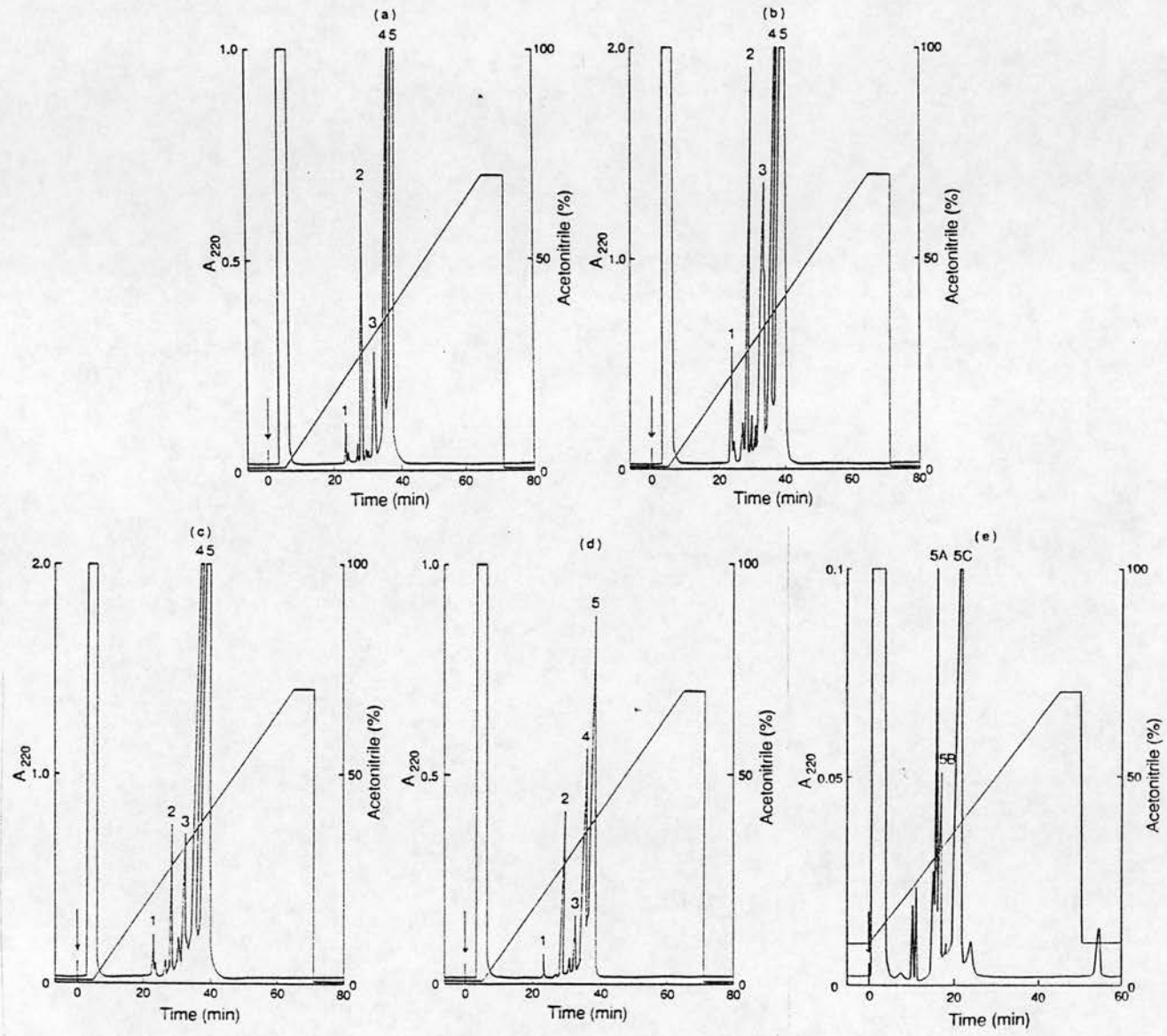


Fig. 3.19. Separation of peptides released by CNBr digestion of the different variants of TRAMP by reverse phase chromatography on an Aquapore RP-300 C8 column

(a) CNBr digest of T2, non-reduced, (b) CNBr digest of T3, non-reduced, (c) CNBr digest of T4, non-reduced, (d) CNBr digest of T5, non-reduced (e) Peak 5 in (b) after reduction with 0.1M DTT. The peaks in (b) were identified by sequence analysis as: (1) TTE, (2) ISY, (3) RGA, (4) pEYG, (5) EWY + PTP + TDY + CR. The peaks in (e) were identified by amino acid analysis and sequence analysis as: (5A) PTP, (5B) TDY and (5C) EWY.

CNBr digest profile were sequenced and identified as the following peptides: (1) TTE, (2) ISY, (3) RGA, (4) pEYG, (5) EWY+PTP+TDY+CR (Fig. 3.23; Tables 3.14 and 3.15). Peak 4 was found to be blocked and later this was found to be the N-terminal peptide by amino acid analysis and mass analysis. Peak 5 yielded several sequences simultaneously. Fractions from peak 5 were collected, dried and redissolved in 6M PBU containing freshly prepared 0.1M dithiothreitol to reduce the disulphide bonds linking the peptides. The reverse phase HPLC profile of this reduced sample is shown in Fig. 3.19e. The peaks were identified by amino acid analysis and sequence analysis as follows: (5A) PTP, (5B) TDY, and (5C) EWY.

3.3.4.4 Clostripain digestion of TRAMP

The T2 to T5 variants of TRAMP were also treated with clostripain and the resulting peptides were separated by reverse phase HPLC (Fig. 3.20a-d). All TRAMP variants gave similar clostripain profiles. The peaks in the T3 clostripain digest profile were sequenced and and identified as the following peptides: (1) GAT, (2) SIF, (3) YFE, (4) QGF, (5) MTD, (6) EWQ and pEYG, (7) AGM, (8) QWK, (9) QWN and CPY, (10) CPY and (11, 12 and 13) clostripain (Fig. 3.23; Tables 3.14 and 3.15).

3.3.4.5 Staphylococcus aureus protease V8 digestion of T3

The elution profile of the T3 protease V8 digest is shown in Fig. 3.21. The following peptides were identified by sequence analysis: (1) SVL + FAN, (2) MDM, (3) YYM, (4) GSD + RDR, (5) V8 protease (Fig. 3.23; Tables 3.14 and 3.15).

3.3.4.6 Sub-digestion of the N-terminal CNBr fragment of T3 with endoproteinase-Asp-N

The T3 CNBr N-terminal fragment (peak 4, Fig. 3.19b), was subdigested with endoproteinase-Asp-N (Fig. 3.22). The following peptides were identified by sequence analysis: (1) DYG, and (2) DDG + DRQ (Fig.

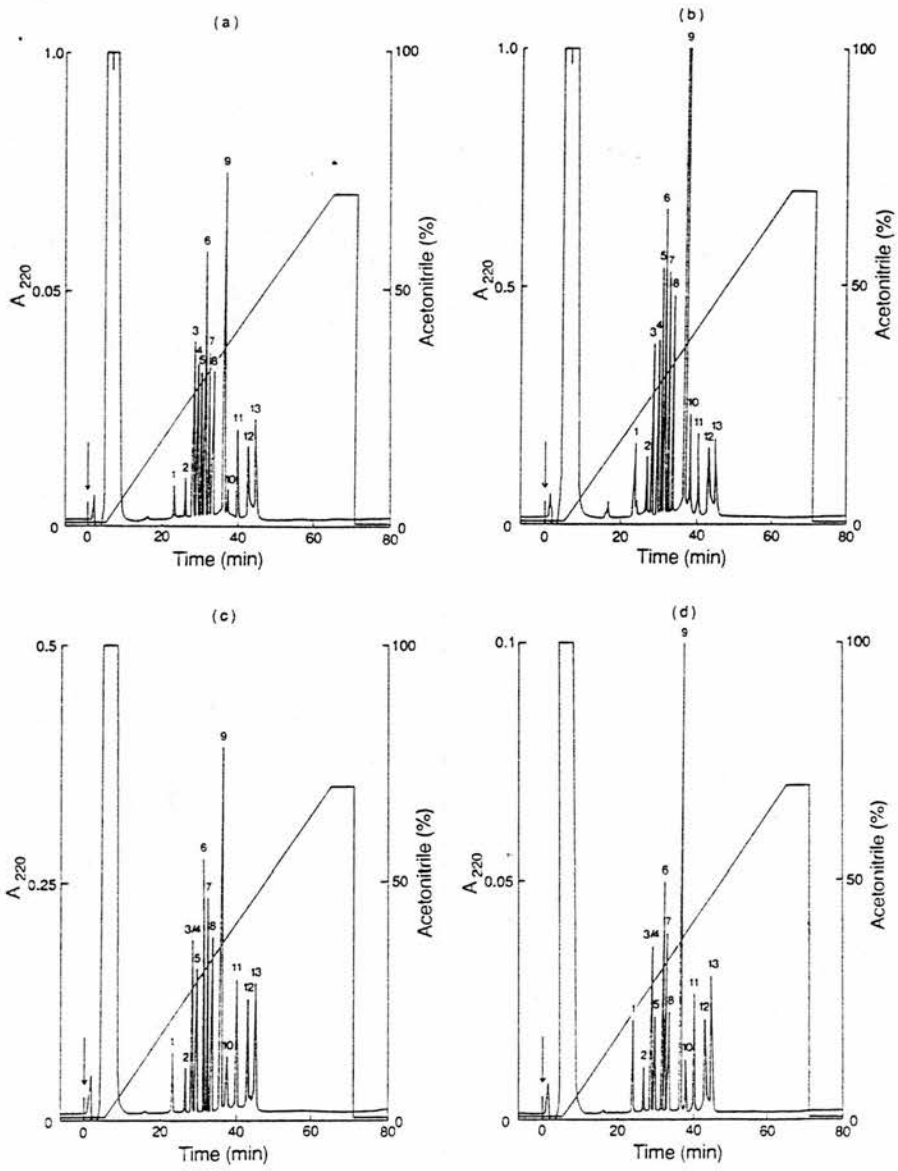


Fig. 3.20. Separation of peptides released by clostripain digestion of the different variants of TRAMP by reverse phase chromatography on an Aquapore RP-300 C8 column

(a) Clostripain digest of T2, (b) clostripain digest of T3, (c) clostripain digest of T4, (d) clostripain digest of T5. These peptides were collected for sequence analysis, and mass analysis on a Biolon 20 mass analyser. The peaks were subsequently found to be as follows: (1) GAT, (2) SIF, (3) YFE, (4) QGF, (5) MTD, (6) EWQ and pEYG, (7) AGM, (8) QWK, (9) QWN and CPY, (10) CPY', (11, 12 and 13) clostripain.

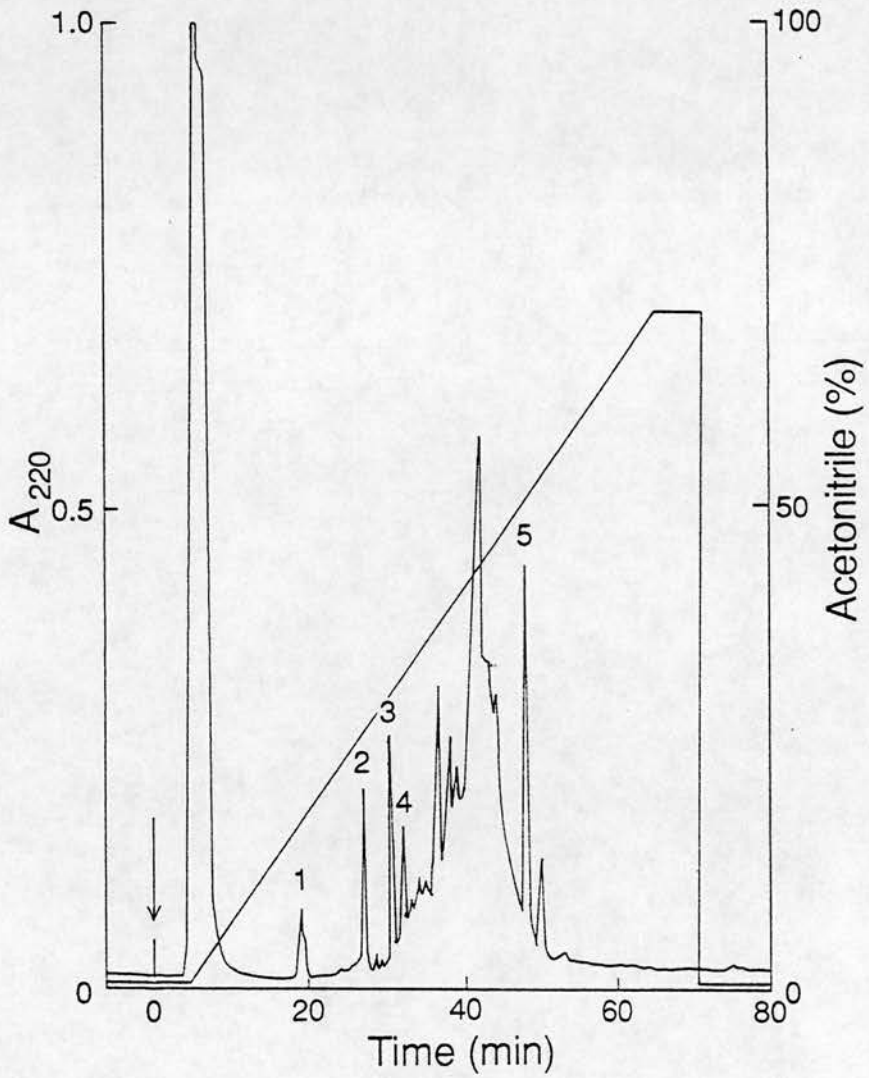


Fig. 3.21. Separation of peptides released by protease V8 digestion of T3 by reverse phase chromatography on an Aquapore RP-300 C8 column

The fractions were subsequently found to be as follows: (1) SVL + FAN, (2) MDM, (3) YYM, (4) GSD and RDR, and (5) protease V8.

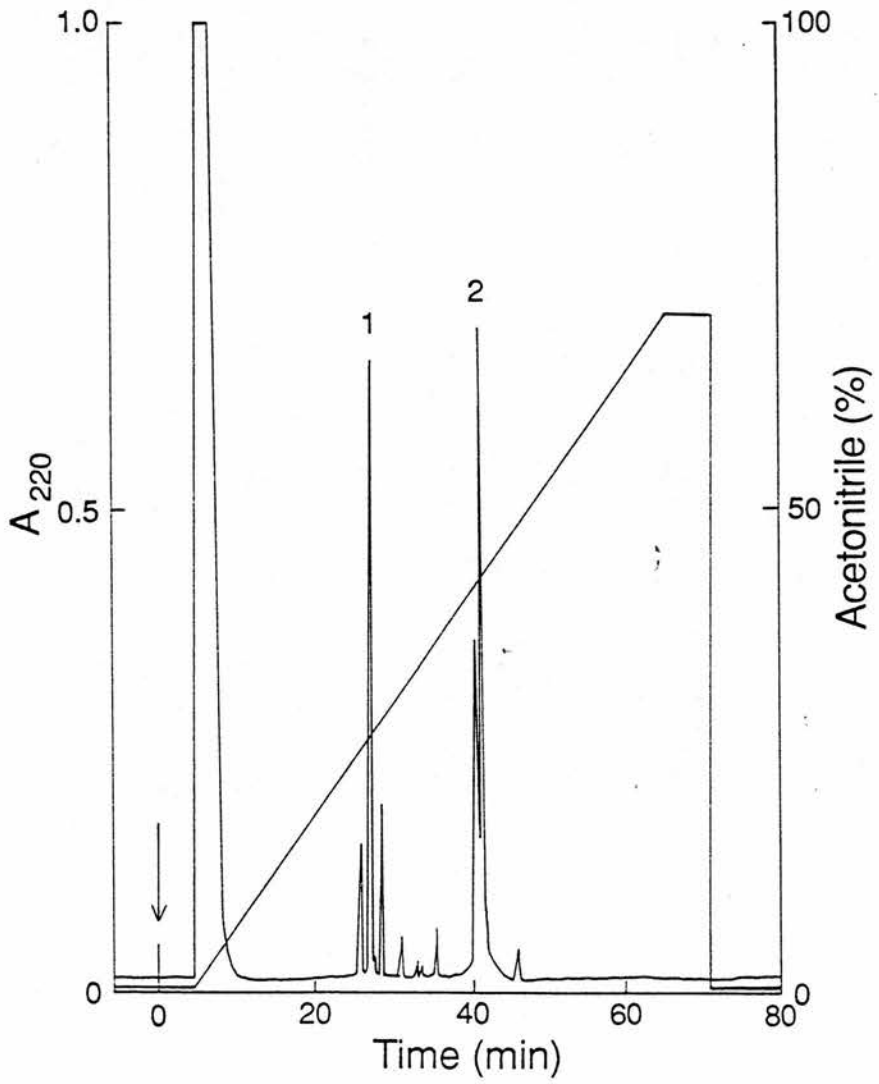


Fig. 3.22. Separation of peptides released by endoproteinase-Asp-N sub-digestion of T3 CNBr peak 4 by reverse phase chromatography on an Aquapore RP-300 C8 column

The fractions were subsequently found to be as follows: (1) DYG, and (2) DDG + DRQ.

3.23; Tables 3.14 and 3.15).

3.3.4.7 Sequence overlap analysis

By overlapping all the sequence data from these various digests the complete sequence of the variant T3 from porcine skin was found (Fig. 3.23). The amino acid sequence of the TRAMP peptides did not match any of the cDNA predicted sequence for lysyl oxidase (Trackman *et al*,. 1990; Trackman *et al.*, 1991). However, when compared (by J.F. Collins, Institute of Cellular and Molecular Biology, Biocomputing Unit, University of Edinburgh) with the Protein Identification Resource database (release 30) using the program "prosrch" (Coulson *et al.*, 1987), the TRAMP sequence was found to be almost identical to the 22K dermatan sulphate proteoglycan-associated extracellular matrix protein from bovine skin (Neame *et al.*, 1989). The sequence of porcine TRAMP differed from the 22K bovine skin protein at only four residues, P8 (S8), Q10 (H10), S70 (T70) and M127 (L127), where the bovine skin residues are shown in parentheses.

As previously observed by Neame *et al.*, (1989), the protein consists of three homologous domains. The repeating sequences of bovine (Neame *et al.*, 1989) and porcine TRAMP are shown in Fig. 3.24. All three domains include a six-residue repeating segment which has a consensus sequence of DR(Q/E)W(N/Q/K)(F/Y).

3.3.5 Mass analysis of intact TRAMP variants

Mass estimation of TRAMP variants by SDS-PAGE gave values of approximately 24K. More precise molecular mass determinations were obtained by mass spectroscopy. Data from the Biolon 20 and Lasermat mass analysers are shown in Fig. 3.25a, Fig. 3.26 and Table 3.16. Based on the complete amino acid sequence of porcine TRAMP (Fig. 3.23; Section 3.3.4), the calculated relative molecular mass in non-reducing conditions is 21,989. The observed relative molecular masses of all TRAMP variants are greater than the calculated value, and the masses increase monotonically by 59Da with decreasing isoelectric point (Fig. 3.27; Table 3.3).

Fig. 3.23. Determination of the sequence of porcine TRAMP

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
BOVINE	PyrE	Y	G	D	Y	G	Y	S	Y	H	Q	Y	H	D	Y	S	D	D	G	W
PyrE	**PyrE**	**Y**	**G**	D	Y	G	Y	**P**	Y	**Q**	Q	Y	H	D	Y	S	D	D	G	W
CNBr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CNBr (red)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clostripain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Asp-N	-	-	-	**D**	**Y**	**G**	Y	**P**	Y	**Q**	Q	Y	H	-	-	-	**D**	**D**	**G**	W

	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
BOVINE	V	N	L	N	R	Q	G	F	S	Y	Q	C	P	H	G	Q	V	V	V	A
PyrE	V	N	L	N	R	Q	G	F	S	Y	Q	C	P	H	G	Q	V	V	V	A
CNBr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CNBr (red)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clostripain	-	-	-	-	-	**Q**	**G**	**F**	S	Y	Q	C	P	H	G	Q	V	V	V	A
V8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Asp-N	V	N	L	N	R	Q	G	F	S	Y	Q	C	P	H	G	Q	V	V	V	A

	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
BOVINE	V	R	S	I	F	N	K	K	E	G	S	D	R	Q	W	N	Y	A	C	M
PyrE	V	R	S	I	F	N	K	K	E	G	S	D	R	Q	W	N	Y	A	C	M
CNBr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CNBr (red)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clostripain	V	R	**S**	**I**	**F**	N	K	K	E	G	S	D	R	**Q**	**W**	**N**	Y	A	C	M
V8	-	-	-	-	-	-	-	-	-	**G**	**S**	**D**	R	Q	W	N	Y	A	-	M
Asp-N	V	R	S	I	F	N	K	K	E	G	S	**D**	**R**	**Q**	W	N	Y	A	C	-

	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
BOVINE	P	T	P	Q	S	L	G	E	P	T	E	C	W	W	E	E	I	N	R	A
PyrE	P	T	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CNBr	**P**	**T**	**P**	Q	S	L	G	E	P	**S**	E	C	W	W	E	E	I	N	R	A
CNBr (red)	**P**	**T**	**P**	Q	S	L	G	E	P	**S**	E	C	W	W	E	E	I	N	R	A
Clostripain	P	T	P	Q	S	L	G	E	P	**S**	E	C	W	W	E	E	I	N	R	**A**
V8	P	T	P	Q	S	L	G	E	-	-	-	-	-	-	-	-	-	-	-	-
Asp-N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
BOVINE	G	M	E	W	Y	Q	T	C	S	N	N	G	L	V	A	G	F	Q	S	R
PyrE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CNBr	G	M	**E**	**W**	**Y**	Q	T	C	S	N	N	G	L	V	A	G	F	Q	S	R
CNBr (red)	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clostripain	**G**	**M**	E	W	Y	Q	T	C	S	N	N	G	L	V	A	G	F	Q	S	R
V8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Asp-N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
BOVINE	Y	F	E	S	V	L	D	R	E	W	Q	F	Y	C	C	R	Y	S	K	R
PyrE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CNBr	Y	F	E	S	V	L	D	R	E	W	Q	F	Y	C	C	R	Y	S	K	R
CNBr (red)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clostripain	**Y**	**F**	**E**	S	V	L	D	R	**E**	**W**	**Q**	F	Y	C	C	R	**Y**	**S**	**K**	R
V8	-	-	-	**S**	**V**	**L**	D	R	E	-	-	-	-	-	-	-	-	-	-	-
Asp-N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
BOVINE	C	P	Y	S	C	W	L	T	T	E	Y	P	G	H	Y	G	E	E	M	D
PyrE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CNBr	C	P	Y	S	C	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CNBr (red)	-	-	-	-	-	-	-	**T**	**T**	**E**	Y	P	G	H	Y	G	E	E	-	-
Clostripain	**C**	**P**	**Y**	S	C	W	M	T	T	E	Y	P	G	H	Y	G	E	E	M	D
V8	**C**	**P**	**Y**	S	C	W	M	T	T	E	Y	P	G	H	-	-	-	-	**M**	**D**
Asp-N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160
BOVINE	M	I	S	Y	N	Y	D	Y	Y	M	R	G	A	T	T	T	F	S	A	V
PyrE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CNBr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CNBr (red)	-	**I**	**S**	**Y**	N	Y	D	Y	Y	M	**R**	**G**	**A**	T	T	T	F	S	A	V
Clostripain	M	I	S	Y	N	Y	D	Y	Y	M	R	**G**	**A**	**T**	T	T	F	S	A	V
V8	**M**	I	S	Y	N	Y	D	**Y**	**Y**	**M**	R	G	A	T	-	-	-	-	-	-
Asp-N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
BOVINE	E	R	D	R	Q	W	K	F	I	M	C	R	M	T	D	Y	D	C	E	F
PyrE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CNBr	-	-	-	-	-	-	-	-	-	-	-	-	-	**T**	**D**	**Y**	D	C	E	F
CNBr (red)	E	R	D	R	Q	W	K	F	I	-	-	-	-	-	-	-	-	-	-	-
Clostripain	E	R	-	-	**Q**	**W**	**K**	F	I	M	C	R	**M**	**T**	**D**	Y	D	C	E	**F**
V8	E	**R**	**D**	**R**	Q	W	K	F	I	M	-	R	M	T	D	Y	D	-	E	F
Asp-N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	181	182	183
BOVINE	A	N	V
PyrE	-	-	-
CNBr	A	N	V
CNBr (red)	-	-	-
Clostripain	**A**	**N**	V
V8	A	N	V
Asp-N	-	-	-

The following symbols are used in the above figure above: - denotes residue not sequenced; + denotes sequence continues. Amino acids in outlined type indicate a residue change in porcine TRAMP compared to the bovine equivalent of TRAMP. Bold letters are used for the first three letters of each peptide sequenced.

Table 3.14. Yields of amino acid residues determined from peptide sequencing of T3

The columns show yields, in pmol, from sequencing different peptides. The columns show, (from left to right): T3 cleaved by 17% methylamine and 3M HCl treatment (ME), pyroglutamate aminopeptidase (PyrE), CNBr unreduced (CNBr) and reduced (CNBr (red)), clostripain (R), protease V8 (E/D) and endoproteinase-Asp-N (D).

Position	Residue	ME	PyrE	CNBr	CNBr (red)	R	E/D	D
1	PyrE	101	**YGD**					
2	Y	208	450					
3	G	192	606					**DYG**
4	D	95	273					?
5	Y	148	568					273
6	G	138	571					315
7	Y	161	565					298
8	**P**	76	438					270
9	Y		394					252
10	**Q**		368					210
11	Q		442					233
12	Y		411					194
13	H		300					18
14	D		250					
15	Y		358					
16	S		110					**DDG**
17	D		199					103
18	D		207					61
19	G		206					110
20	W		68					91
21	V		171					84
22	N		157					82
23	L		111					69
24	N		146					23
25	R		107			**QGF**		2
26	Q		130			98		60
27	G		155			126		64
28	F		121			163		52
29	S		49			99		24
30	Y		146			103		41
31	Q		80			101		37
32	C		74			99		33
33	P		86			70		43
34	H		82			23		9
35	G		86			65		35
36	Q		67			61		25
37	V		75			41		24
38	V		81			57		28
39	V		95			56		32
40	A		63			49		20
41	V		78			38		22
42	R		50			17		11
43	S		23			67**SIF**		5
44	I		36			198		10
43	F		43			154		10

Position	Residue	PyrE	CNBr	CNBr (red)	R	E	D
46	N	39			94		9
47	K	4			78		2
48	K	5			88		1
49	E	39			63	**GSD**	3
50	G	46			110	120	2
51	S	17			20	57	2
52	D	20			41	73	103 **DRQ**
53	R	24			14	28	14
54	Q	29			255 **QWN**	73	108
55	W	13			129	23	91
56	N	24			265	47	84
57	Y	47			259	50	84
58	A	22			244	51	61
59	C	26			110	-	20
60	M	19	**PTP**	**PTP**	322	46	?
61	P	25	270	189	130	50	
62	T	12	478	118	126	63	
63	P	26	488	119	125	46	
64	Q		1285	80	80	26	
65	S		127	24	28	14	
66	L		302	54	71	20	
67	G		329	48	85	21	
68	E		261	38	35	21	
69	P		306	69	55		
70	**S**		91	17	18		
71	E		316	29	64		
72	C		184	6	8		
73	W		60	11	7		
74	W		73	14	7		
75	E		166	9	19		
76	E		210	8	21		
77	I		42	4	18		
78	N		51	3	37		
79	R		117	0.4	2		
80	A		46	3	490 **AGM**		
81	G		88	3	446		
82	M		**EWY**		417		
83	E		853		272		
84	W		873		79		
85	Y		1522		213		
86	Q		1285		289		
87	T		982		142		
88	C		1003		152		
89	S		415		85		
90	N		725		223		
91	N		854		186		
92	G		753		132		
93	L		679		123		
94	V		698		113		
95	A		579		109		
96	G		593		99		
97	F		543		91		
98	Q		416		76		
99	S		171		29		
100	R		286		20		
101	Y		414		207 **YFE**		

Position	Residue	PyrE	CNBr	CNBr (red)	R	E	D
102	F		337		242		
103	E		204		110	SVL	
104	S		101		164	88	
105	V		223		175	497	
106	L		208		169	451	
107	D		136		150	240	
108	R		168		39	123	
109	E		96		340	EWQ 87	
110	W		56		174		
111	Q		90		456		
112	F		104		506		
113	Y		133		356		
114	C		108		332		
115	C		124		369		
116	R		81		29		
117	Y		97		201	YSK	
118	S		29		118		
119	K		20		151		
120	R		48		17	CPY'	
121	C		59		CPY 50	5	
122	P		48		202	14	
123	Y		58		168	9	
124	S		18		76	6	
125	C		39		67	7	
126	W		19		50	9	
127	M			TTE	322	6	
128	T			247	60	3	
129	T			219	126	4	
130	E			166	45	5	
131	Y			176	60	5	
132	P			256	115	8	
133	G			161	62	+	
134	H			34	16		
135	Y			97	40		
136	G			90	80		
137	E			47	51		
138	E			52	64	MDM	
139	M			?	26	245	
140	D				31	262	
141	M			ISY	28	279	
142	I			3956	38	369	
143	S			1456	13	135	
144	Y			2169	26	284	
145	N			1998	37	234	
146	Y			1904	26	275	
147	D			1255	16	46	
148	Y			1697	25	80	YYM
149	Y			1442	26	90	
150	M			RGA	11	74	
151	R			49	8	25	
152	G			536	51	GAT 85	
153	A			528	52	85	
154	T			327	22	43	
155	T			314	19	ND	
156	T			326	16	ND	

Position	Residue	PyrE	CNBr	CNBr (red)	R	E	D
157	F			321	20		
158	S			139	8		
159	A			298	18		
160	V			263	15		
161	E			126	9	**RDR**	
162	R			150	1	12	
163	D			132		57	
164	R			121	**QWK**	32	
165	Q			114	186	149	
166	W			8	53	64	
167	K			17	154	108	
168	F			21	135	121	
169	I			6	146	126	
170	M			?	125	85	
171	C				116	-	
172	R				36	47	
173	M		**TDY**		343 **MTD**	79	
174	T		237		168	63	
175	D		137		184	39	
176	Y		152		289	56	
177	D		235		201	37	
178	C		190		273	-	
179	E		239		139	17	
180	F		449		210	433**FAN**	
181	A		167		234	690	
182	N		854		205	379	
183	V		293		81	256	

The following symbols are used in the above table: ? denotes residue unknown; + denotes sequence continues; - denotes blank cycle due to oxidation of cysteine to cysteic acid.

Table 3.15. Repetitive yields of T3 peptides sequenced

	N-terminus	Repetitive Yield (%)	Residues used
i.			
	EYG	95	Y
	YGD	92	G,D,Y,P,Q,S,V,N
ii.	Unreduced CNBr peptides		
	PTP	90	
	EWY	90	W,V,G
	TDY	90	
iii.	Reduced CNBr peptides		
	PTP	91	P,S,E
	TTE	86	Y
	ISY	87	Y
	RGA	88	A,T,R
iv.	Clostripain peptides		
	QGF	95	G,V
	SIF	98	*
	QWN	91	P,Y
	AGM	88	Q,S,N,A,G
	YFE	96	*
	EWQ	88	*
	YSK	—	
	CPY	91	P,Y
	CPY'	93	P,Y
	GAT	84	A,T
	QWK	87	*
	MTD	87	*
v.	Protease V8 peptides		
	GSD	90	G,P,S,Q
	SVL	98	*
	MDM	97	Y
	YYM	90	*
	RDR	97	D
	FAN	98	*
vi.	Endoproteinase-Asp-N peptides		
	DYG	95	Y
	DDG	97	V,Q,G
	DRQ	97	*

The symbols are as follows: * = Repetitive yield calculated from a different run made on the same day because peptide contained no repeat residues.

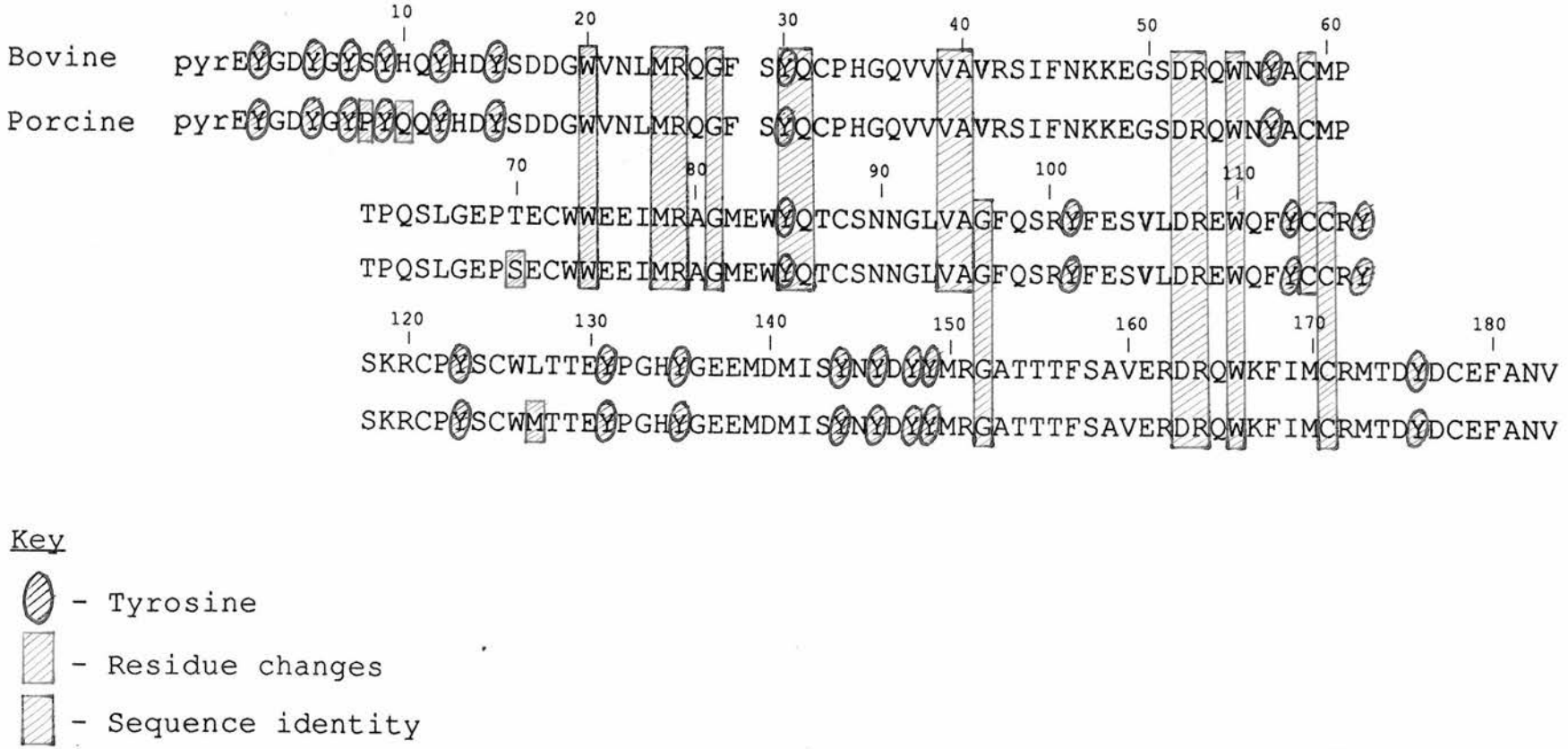


Fig. 3.24. Comparison of amino acid sequences of bovine (Neame *et al.*, 1989) and porcine TRAMP

The sequence contains three homologous domains which are aligned for maximum similarity.

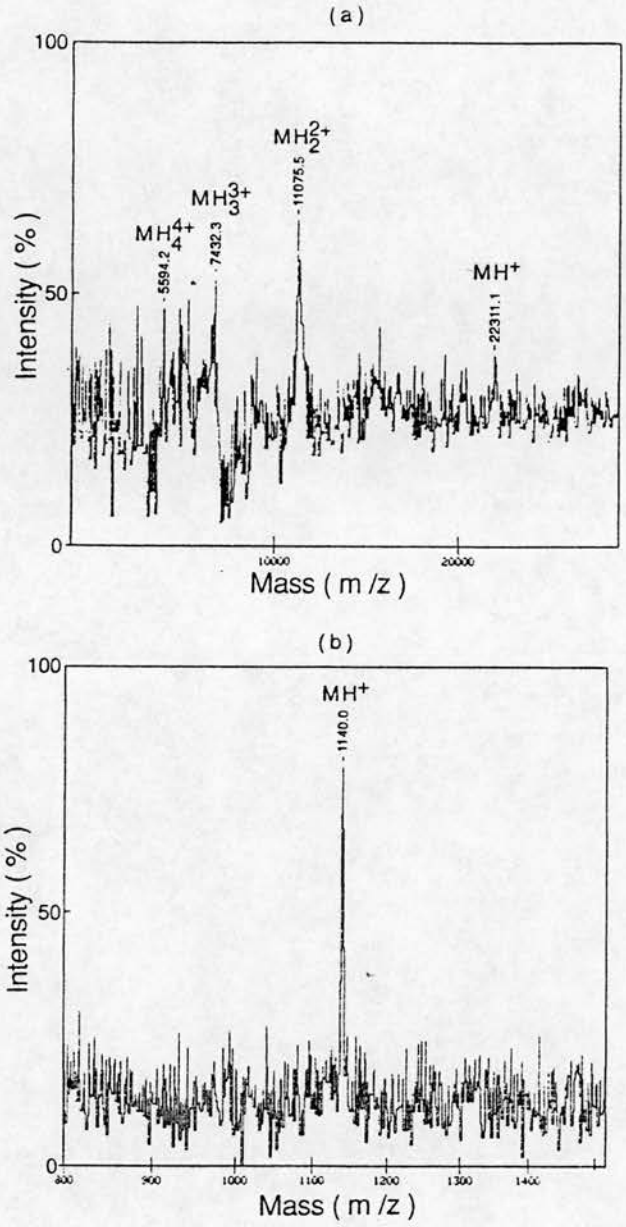


Fig. 3.25. Spectra of T3 and T3 clostripain peptides from a Biolon 20 plasma desorption mass analyser

(a) Approximately 1 nmol T3 was analysed. The mass of T3 was found by taking the mean of MH^+ = 22,310, MH_2^{2+} = 22,251, MH_3^{3+} = 22,294 and MH_4^{4+} = 22,373. This gave a mean M_r = 22, 307. The MH^{2+} ion often gives the strongest signal for proteins and peptides in the high mass range in plasma desorption mass spectrometry (P. Hojrup, personal communication).

(b) Approximately 10 pmol of the peptide T3CL1 (i.e. peak 1 from a clostripain digest of T3 (Fig. 3.20b) was analysed. The peptide was found to be GAT which had a theoretical M_r = 1139.3.

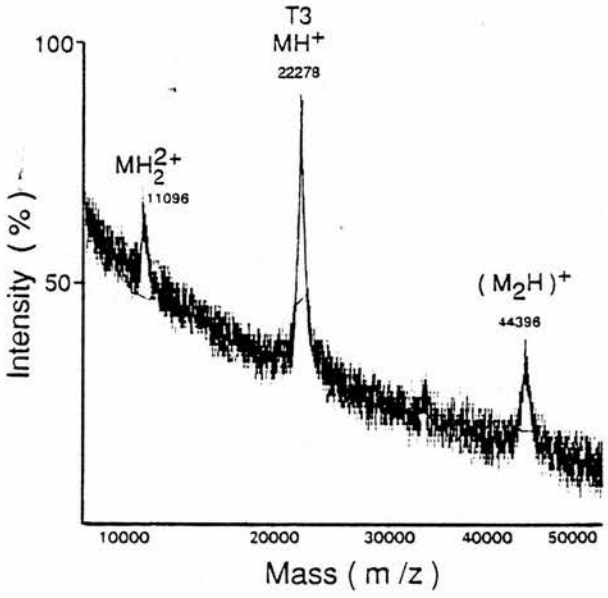


Fig. 3.26. Spectra of T3 from a Finnigan MAT Lasermat laser desorption mass analyser

Approximately 100 pmol T3 was analysed.

Table 3.16. Molecular masses of TRAMP variants

Variant	Biolon 20[a]	Lasermat[b]
T1	N.D.	22,119 +/- 22
T2	N.D.	22,183 +/- 22
T3	22,307 +/- 230	22,252 +/- 22
T4	22,147 +/- 230	22,306 +/- 22
T5	22,616 +/- 230	22,352 +/- 22

ND = not determined.

[a] Biolon 20 values for proteins between 20,000 and 30,000 are accurate to +/-1% as the instrument is working at the limit of its range.

[b] Errors are standard deviations based on lysyl oxidase samples (n = 8), using carbonic anhydrase (M_r = 29,024) as an external standard.

The mass of the T3 sequence (M_r = 21,989; Cronshaw *et al.*, 1993) compares with M_r = 21,984 for the bovine equivalent of TRAMP (Neame *et al.*, 1989).

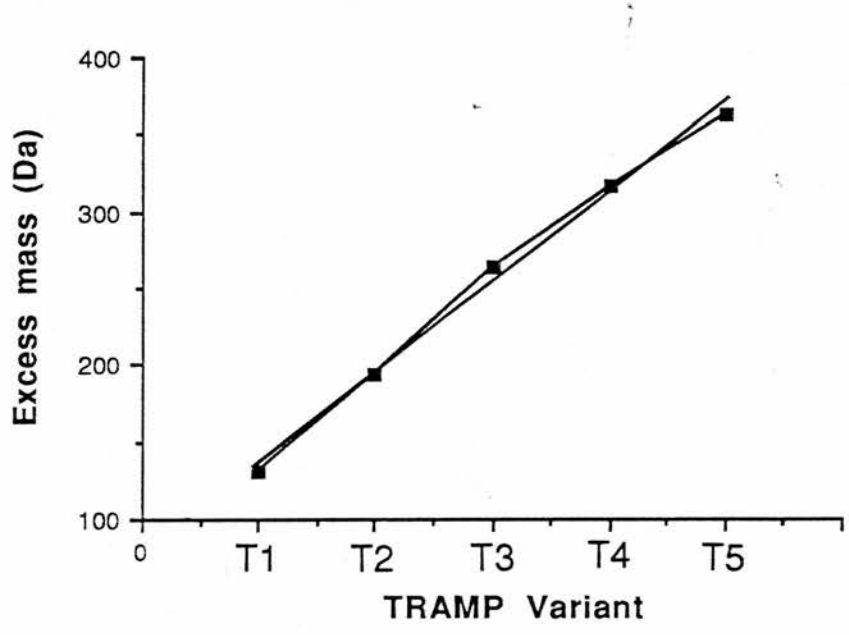


Fig. 3.27. Graph showing incremental mass changes between TRAMP variants

The incremental mass difference between variants is 59Da.

The differences in the observed and calculated molecular masses and isoelectric point data suggest that some amino acid residues in TRAMP may be modified at the post-translational level. The modification(s) would be acid labile, to account for the apparent absence of modified amino acids by sequence or amino acid analysis, and would increase the net negative charge, to account for the low pI. The presence of an acidic post-translational modification was suggested by the observation (J.R.E. MacBeath) that TRAMP stains strongly with Alcian Blue (Fig. 3.28; Cronshaw *et al.*, 1993).

3.3.6 Mass analysis of T3 and T4 clostripain peptides and T3 CNBr peptides

Peptides from T3 and T4 clostripain digests were collected after reverse phase HPLC and subjected to mass analysis using a Biolon 20 mass analyser (Fig. 3.25b; Table 3.17). The values obtained confirm the sequence data but do not indicate any differences between the two variants.

Three T3 CNBr peptides were also investigated by mass analysis (Table 3.18). These help to confirm the sequence data, but are less accurate than clostripain peptide values because of the difficulty in determining whether the peptide is in the homoserine (hs) or homoserine lactone (hsl) form (Ambler, 1965).

The masses of the following T3 clostripain peptides were fed into the Molecular Weight Search (MOWSE) database (by D. Pappin, Imperial Cancer Research Fund, London): pyrEYG (3,108), QGF (1,876), QWN (3,155), AGM (2,319), YFE (1,028), EWQ (1,135), GAT (1,139), and MTD (1,305). Other data supplied to the database included the approximate molecular mass of TRAMP (22K), the cleavage reagent, Arg-C, and a mass error tolerance of 2Da. These data were then scanned against 51,093 sequences in the database which resulted in the correct sequence alignment with the 22K bovine protein (Neame *et al.*, 1989). No sequence match was found for peptides that contained sequence substitutions between porcine and bovine TRAMP e.g. pyrEYG and QWN. When the masses of the T3 CNBr peptides pyrEYG (7,267), ISY (1,183) and RGA (2,354) were scanned in the same database, sequence alignment was also found with the 22K bovine protein (Neame *et*

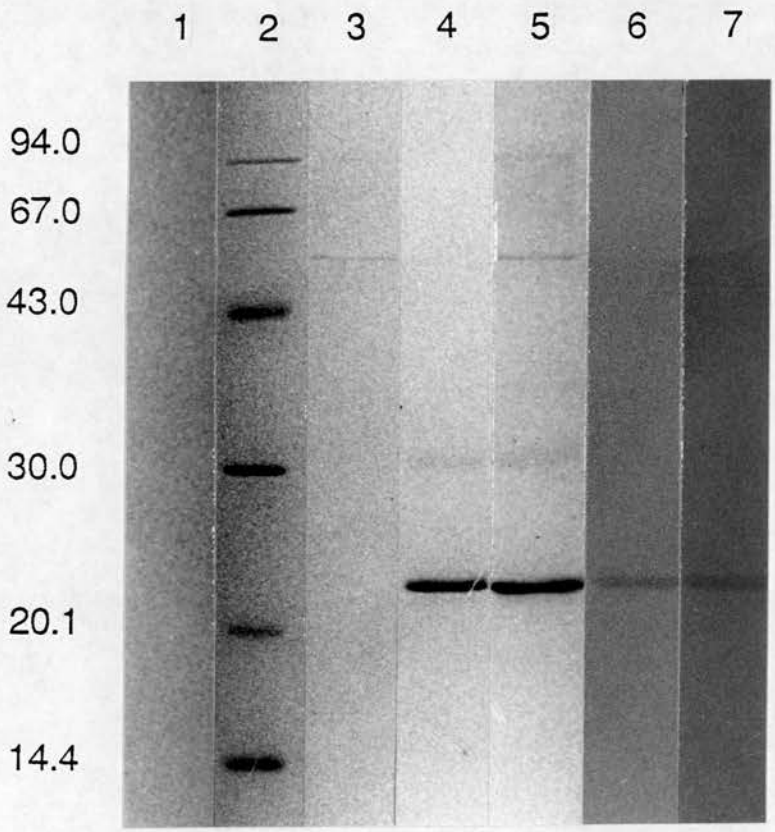


Fig. 3.28. SDS-PAGE analysis of combined forms of TRAMP after treatment with aryl-sulphatase

Approximately 1ug of each reduced sample was run on a 12% discontinuous SDS-PAGE gel. The lanes are as follows: (1) blank, (2) protein standards, (3) aryl-sulphatase, (4) TRAMP, (5) TRAMP + aryl-sulphatase, (6) TRAMP, (7) TRAMP + aryl-sulphatase. Lanes (1-5) were stained with Coomassie Blue, whilst lanes (6-7) were stained with Alcian Blue. The molecular masses (K) of protein standards are indicated.

Table 3.17. Comparison of mass values of T3 and T4 clostripain peptides using a Biolon 20 mass analyser

Peptide	Sequence M_r	T3 Biolon M_r	T4 Biolon M_r
PyrEYG	3100.7	3108.2	ND
QGF	1875.5	1875.4	1874.9
SIF	1480.8	ND	1479.3
QWN	3151.7	3154.8	3151.9
	+16 = 3167.7		
AGM	2320.0	2319.4	2319.1
	+16 = 2336.0		
YFE	1028.2	1027.9	1027.5
EWQ	1134.5	1134.9	1134.1
YSK	651.7	ND	ND
CPY	3820.9		
	+64 = 3884.9	3890.8	3891.8
GAT	1139.3	1139.0	1138.6
QWK	1111.6		
	+16 = 1127.6	1128.4	1129.0
MTD	1307.5	1305.1	ND
	+16 =1323.5	1319.7	

The table shows a comparison of observed mass values for peptides from TRAMP variants T3 and T4 (Figs. 3.20b, c), produced by clostripain digestion, with theoretical mass values. Methionine is oxidised by this method to methionine sulphoxide (MSO) thus 16 has been added to the predicted masses of methionine containing peptides. The Biolon values shown have an error of +/- 0.1% for peptides. ND = not determined.

Table 3.18. Mass values of T3 CNBr peptides using a Biolon 20 mass analyser

Peptide	Sequence M_r	T3 Biolon M_r
pyrEYG (hs)	7296.2	7287.6
pyrEYG (hsl)	7278.2	7266.6
ISY (hs)	1211.5	1207.0
ISY (hsl)	1193.5	1183.3
RGA (hs)	2381.0	2378.9
RGA (hsl)	2363.0	2354.0

Mass values from other T3CNBr peptides were not determined because of the difficulty in determining their precise masses due to the presence of homoserine (hs) and homoserine lactone (hsl) forms of the peptides (Ambler, 1965).

al., 1989). No sequence match was found for peptide pyrEYG as a residue change occured at residues 8 and 10. Remarkably the correct sequence was identified from just two CNBr peptide masses and no amino acid sequence information.

3.3.7 Glycosylation

Possible glycosylation of TRAMP was investigated as follows. By amino acid analysis, there was no evidence for hexosamines in TRAMP, as also observed by Neame *et al.*, (1989). Derivatised galactosamine (galN) and glucosamine (glcN) elute after PTC-Glu and before PTC-Ser, respectively, when separated on an Applied Biosystems PTC C-18 column (Fig. 2.1). Chemical deglycosylation of T3 variant of TRAMP with trifluromethane sulphonic acid (TFMS) did not show any decrease in molecular mass after measurement by LDMS. Furthermore, enzymic deglycosylation of TRAMP variants (by J.R.E. MacBeath) showed no change in electrophoretic mobility or band sharpness by SDS-PAGE (Cronshaw *et al.*, 1993).

3.3.8 Tyrosine sulphation

One possible post-translational modification was tyrosine sulphation. There are five possible tyrosine residues in TRAMP that meet four of the five empirically determined rules for tyrosine sulphation (Hortin *et al.*, 1986; Section 4.4.3).

The tyrosine sulphate ester bond is particularly labile to acid hydrolysis (Huttner, 1984). Following treatment with 1M HCl at 100°C for 5 min, Alcian Blue staining of TRAMP almost halved relative to Coomassie Blue staining (Cronshaw *et al.*, 1993, with permission of J.R.E. MacBeath). This is consistent with the observation that acid hydrolysis of TRAMP did not yield any sulphotyrosine after amino acid composition analysis.

The combined forms of TRAMP were treated with and without aryl-sulphatase and then run in duplicate on a 12% SDS-PAGE gel. The gels were stained with Coomassie Blue or Alcian Blue (Fig. 3.28). After treatment with aryl-sulphatase the TRAMP band still stained with Coomassie Blue, but

Alcian Blue staining was reduced. To further investigate possible tyrosine sulphation, the T3 variant of TRAMP was examined on a Mono Q column before and after treatment with 0.1 unit of aryl-sulphatase at 37°C for 2h. The results (Fig. 3.29) show that while a small protein peak continued to be present, after treatment with sulphatase, at the original elution position of T3, three additional peaks (T0', T1', T2') appeared earlier in the elution gradient. The elution positions of two of these peaks coincided with T1 and T2. When examined by SDS-PAGE (Fig. 3.30) however, all three peaks co-migrated with T3.

Various attempts were made to identify sulphotyrosine in TRAMP by high voltage paper electrophoresis (HVPE) and amino acid analysis. A standard of sulphotyrosine (prepared by J.R.E. MacBeath) was found to separate from tyrosine by HVPE at pH 6.5 (Fig. 3.31). PTC-sulphotyrosine was found to elute between proline and PTU on a PTC C-18 column (Fig. 2.1). The T3 variant of TRAMP was hydrolysed under alkaline conditions with 0.2M $Ba(OH)_2$ at 100°C for 22h (Huttner, 1984). The sample was then neutralised with 0.1M H_2SO_4, the precipitate removed by centrifugation, and the supernatant analysed by both HVPE and amino acid analysis. No visible sulphotyrosine was seen, by either method, in the T3 alkaline hydrolysate. When sulphotyrosine standard was subjected to alkaline hydrolysis with $Ba(OH)_2$ and run on HVPE, sulphotyrosine was still present but there was some hydrolysis of the ester bond to form tyrosine (Fig. 3.31, Lane 5). The absence of sulphotyrosine in the T3 alkaline hydrolysate could be explained either by destruction of the sulphotyrosine ester bond during hydrolysis or by having insufficient material. Overloading a T3 alkaline hydrolysate on the HVPE did not reveal any tyrosine sulphate (data not shown).

There is a distinct spot which is more positively charged in the 0.2M $Ba(OH)_2$ digest of T3, which does not show up in the standard (Fig. 3.31, Lane 2). This is ornithine formed on alkaline hydrolysis.

3.3.9 Cell culture and immunoprecipitation of [^{3}H]- and [^{35}S]-labelled TRAMP

The biosynthesis of TRAMP was investigated using human foreskin

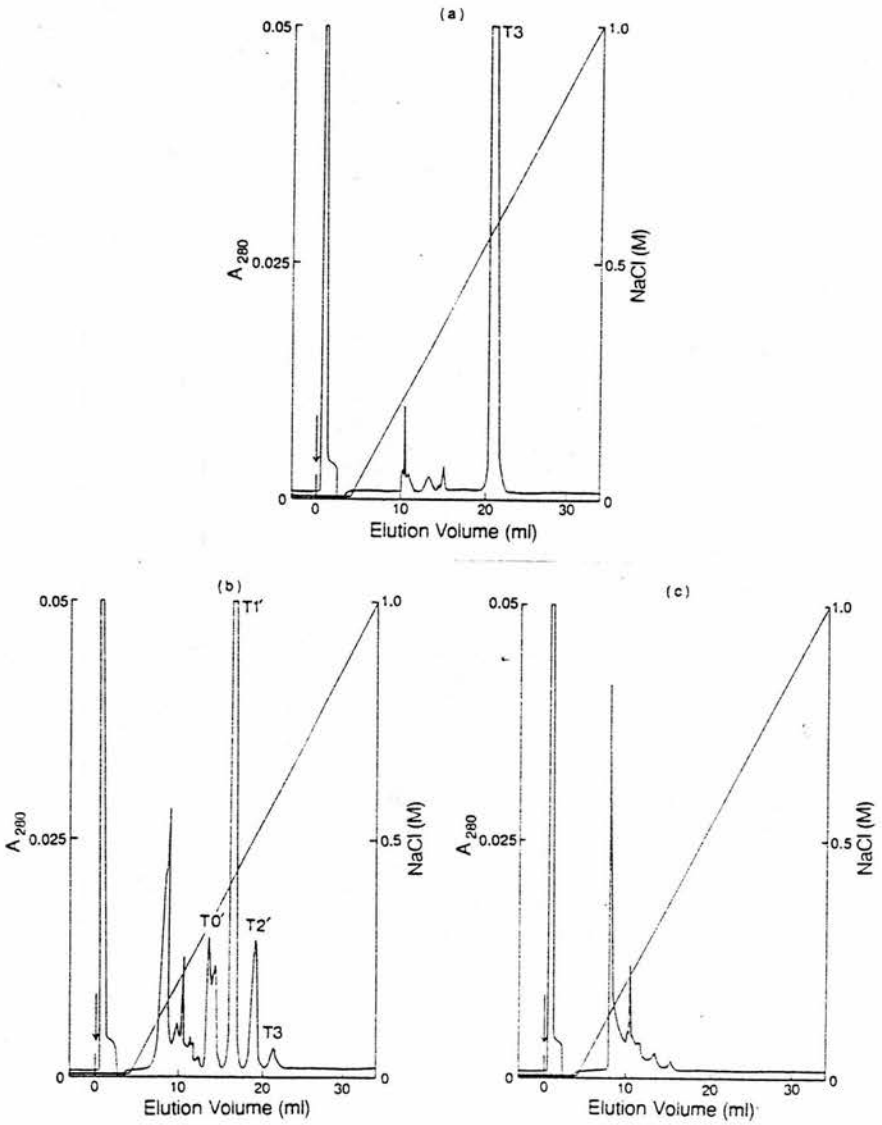


Fig. 3.29. Separationof T3 after digestion with aryl-sulphatase by anion exchange chromatography

T3 following purification by Mono Q chromatography was dialysed and digested by aryl-sulphatase and re-loaded onto the Pharmacia Mono Q HR 5/5 column.

(a) T3, undigested, (b) T3 after digestion with aryl-sulphatase, (c) aryl-sulphatase.

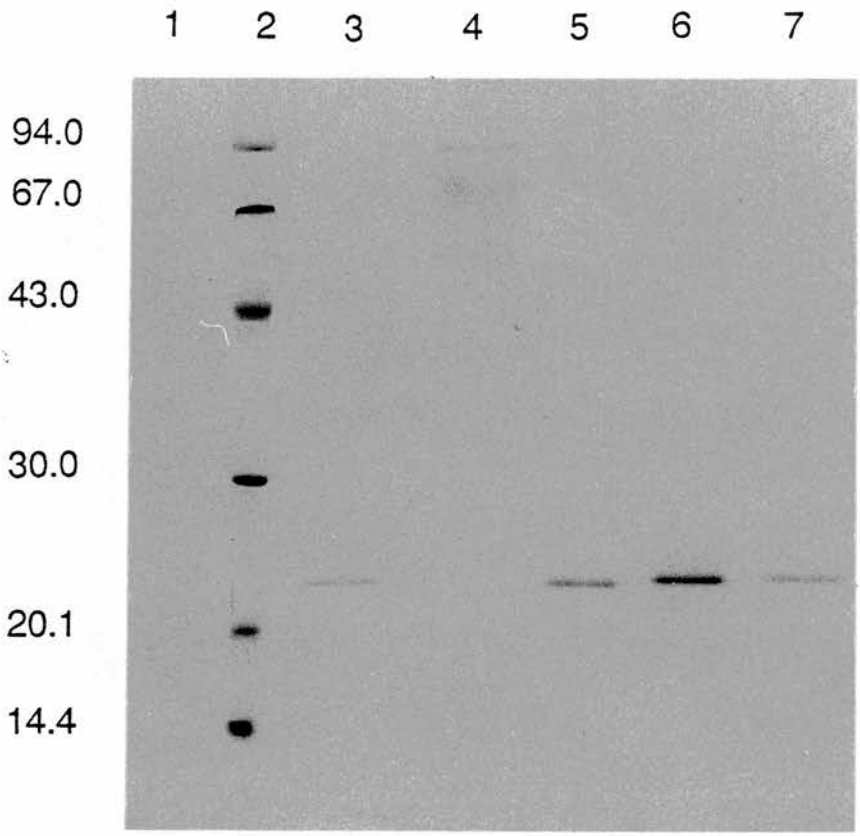


Fig. 3.30. SDS-PAGE analysis of Mono Q fractions of T3 before and after treatment with aryl-sulphatase

Approximately 1µg of each sample was run on a 12% SDS-PAGE gel. The lanes are as follows: (1) blank, (2) protein standards, (3) T3, undigested, (4) aryl-sulphatase, (5) T0', (6) T1', (7) T2' (Fig. 3.29). The gel was stained with Coomassie Blue. The molecular masses (K) of protein standards are indicated.

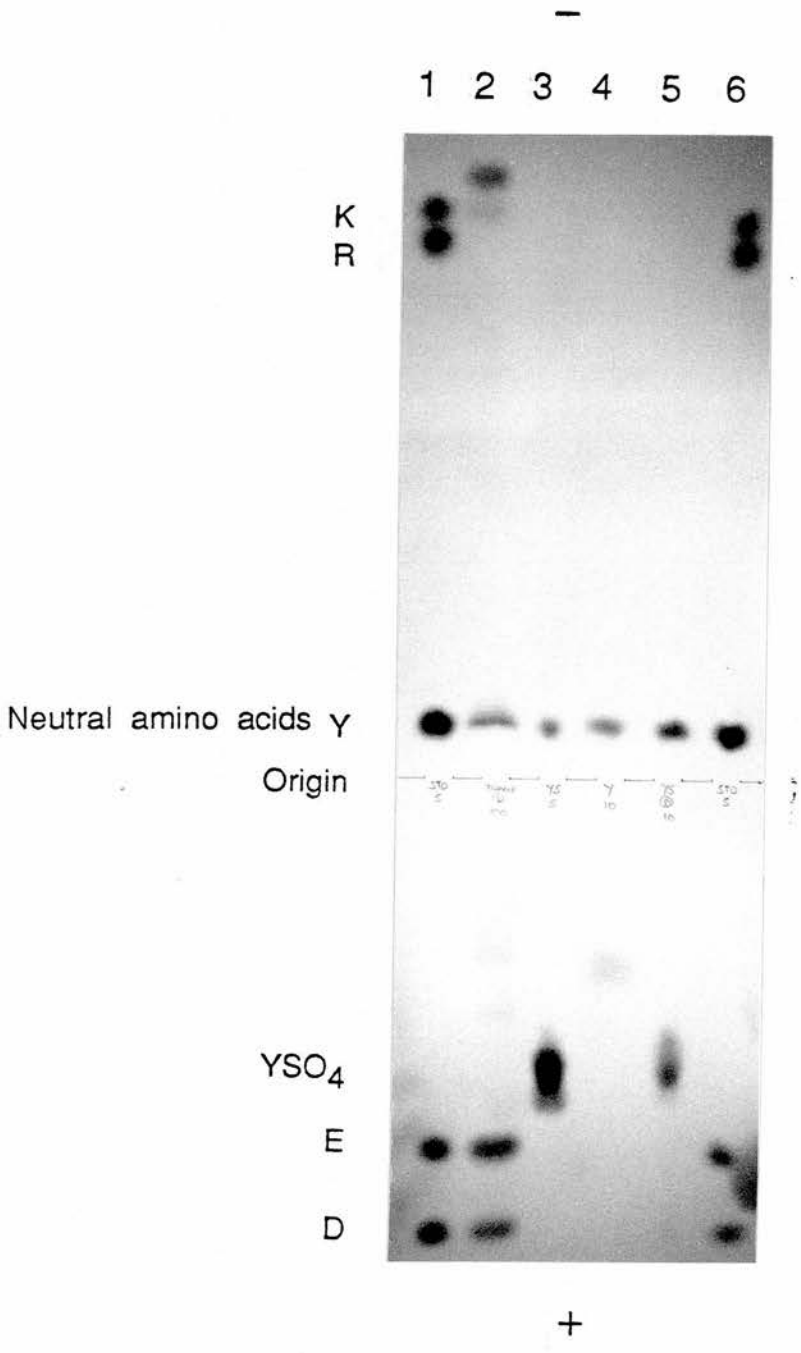


Fig. 3.31. Separation of tyrosine and tyrosine sulphate by high voltage paper electrophoresis (HVPE) in pH 6.5 buffer

The lanes were identified as follows: (1) amino acid standard, (2) TRAMP after 0.2M $Ba(OH)_2$ digestion (3) sulphotyrosine (YSO_4) containing some tyrosine, (4) tyrosine, (5) sulphotyrosine after 0.2M $Ba(OH)_2$ digestion, (6) amino acid standard.

fibroblast cell cultures. The fibroblasts were incubated in the presence of [^{3}H]tyrosine for 24h. The culture media and cell lysates were immunoprecipitated by sequential exposure to killed *Staph. A.* which was pre-loaded with either pre-immune serum or anti-TRAMP anti-serum. Subsequent analysis by SDS-PAGE and fluorography (by U.H. Genschel and F. Simpson; Fig. 3.32a) showed that [^{3}H]tyrosine was incorporated into a protein that was specifically precipitated by the anti-TRAMP anti-serum. The protein migrated at the same position as [^{14}C]-labelled purified porcine TRAMP (Fig. 3.32a; Lane 2), and was mainly present in the culture media (Lane 3) with only small amounts detected in the cell lysate (Lane 4).

Fibroblasts were also cultured in the presence of ^{35}S-sulphate. As with the [^{3}H]tyrosine label, a ^{35}S-labelled protein that co-migrated with [^{14}C]TRAMP standard was specifically precipitated with the anti-TRAMP anti-serum (Fig. 3.32b, lane 3) and this protein was mainly present in the medium.

Having shown that TRAMP secreted by human fibroblasts incorporated both [^{3}H]tyrosine and ^{35}S-sulphate, the sample was transfered from the SDS-PAGE gel, hydrolysed and analysed by HVPE and amino acid analysis in order to detect radiolabelled sulphated tyrosine. Several attempts were made to elute [^{3}H]- or [^{35}S]-labelled TRAMP from the SDS-PAGE gels of the immunoprecipitates. The first technique was electroelution. TRAMP bands, after addition of unlabelled TRAMP to the samples, were identified in the gels by Coomasie Blue staining. Bands were then cut out of the gels and placed into an electroelution device. However, according to the recovery of c.p.m., even after extending the elution time by several hours, the majority of the sample still remained within the gel. As this method proved unsuccessful, electroblotting on to ProBlott membranes was used. The blotted bands were stained with Coomassie Blue and cut out for alkaline hydrolysis. Blotting was found to be 80% efficient and this was the method of choice.

The alkaline hydrolysates of [^{3}H]tyrosine and ^{35}S-sulphate labelled TRAMP were separated by HVPE. In order to detect any labelled amino acids, the Whatman 3MM HVPE paper was divided into 1 cm^{2} areas which were cut out and scintillation counted (by F. Simpson). The total recovery of cpm after HVPE was low (<3%) with losses believed to have occurred during the electrophoresis process. A major source of this loss was thought to be water

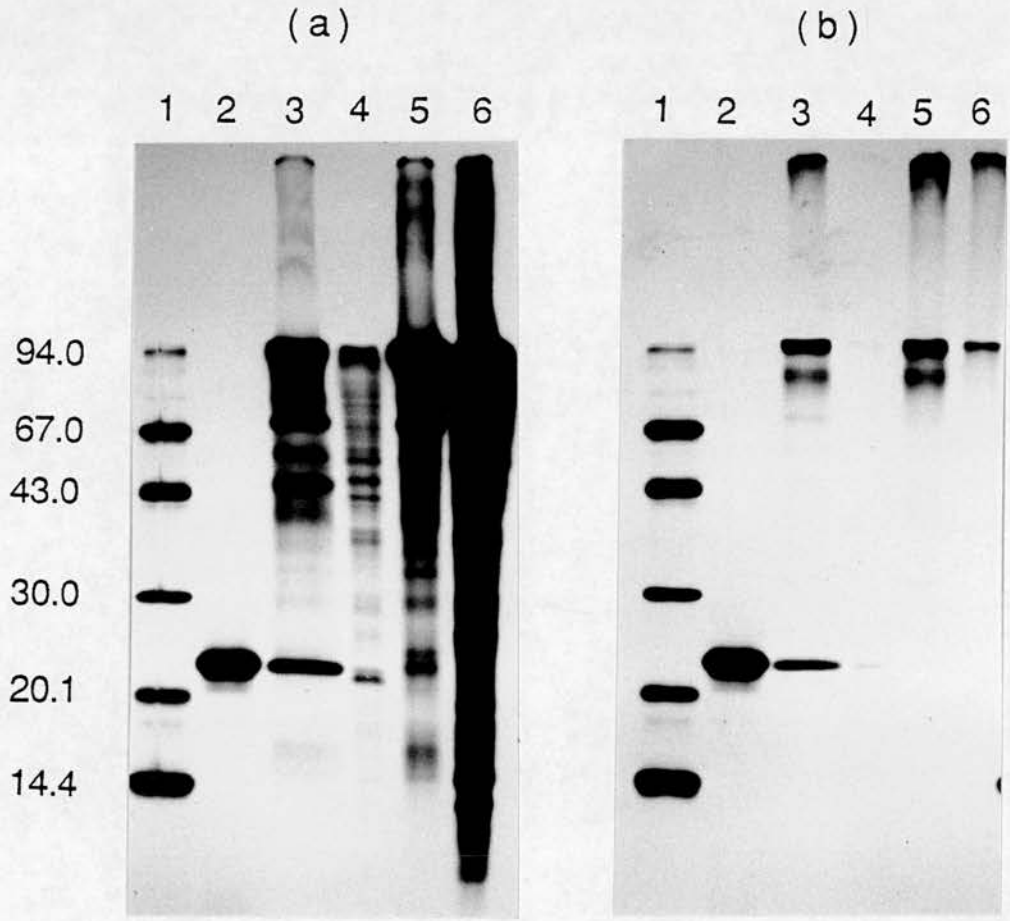


Fig. 3.32. Immunoprecipitation of (a) [^{3}H]tyrosine-labelled TRAMP and (b) ^{35}S-sulphate labelled TRAMP from human fibroblast cultures

Lanes are as follows: (1) ^{14}C-labelled molecular weight markers, (2) ^{14}C-labelled TRAMP standard, (3) culture medium, anti-TRAMP antiserum, (4) cell lysate, anti-TRAMP antiserum, (5) culture medium, pre-immune serum, (6) cell lysate, pre-immune serum. The molecular masses (K) of protein standards are indicated.

contamination of the toluene coolant in the Michl tank during HVPE, which could have dissolved the hydrolysate from the Whatman 3MM paper into the coolant.

In an attempt to improve recovery, the alkaline hydrolysates of [^{3}H]tyrosine and ^{35}S-sulphate labelled TRAMP were separated on an amino acid analyser. Sulphotyrosine and tyrosine were found to be well resolved using the PTC C-18 column (Section 2.6.8; Fig. 3.33a-c). Fifteen second fractions from the eluant of the PTC C-18 column were collected and scintillation counted. The alkaline hydrolystate of ^{35}S-sulphate labelled TRAMP showed a distinct peak in the position of sulphotyrosine. The hydrolysates of [^{3}H]tyrosine labelled TRAMP showed two clear peaks one in the position of sulphotyrosine and the other in the position of tyrosine (Fig. 3.33d and e).

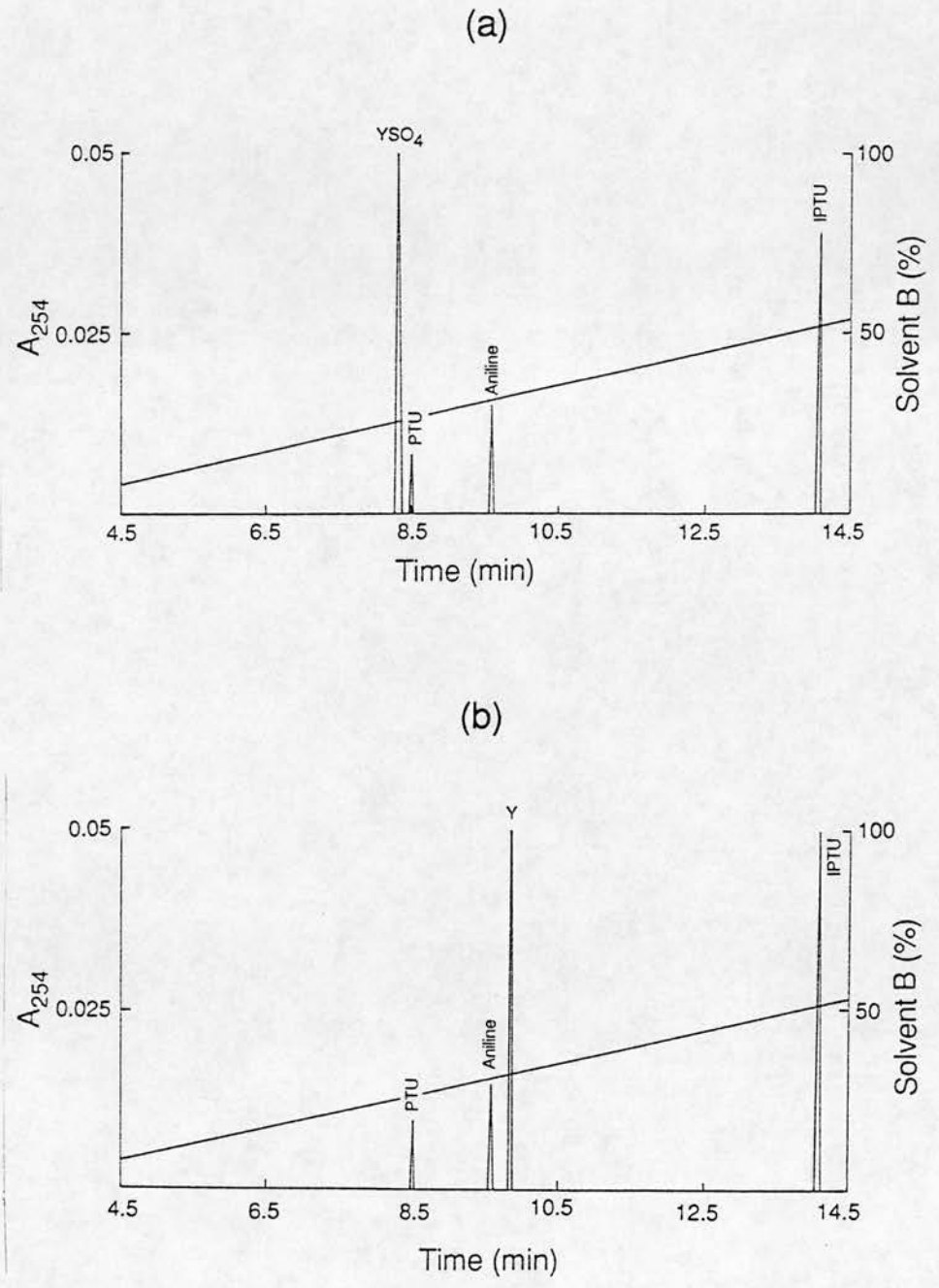
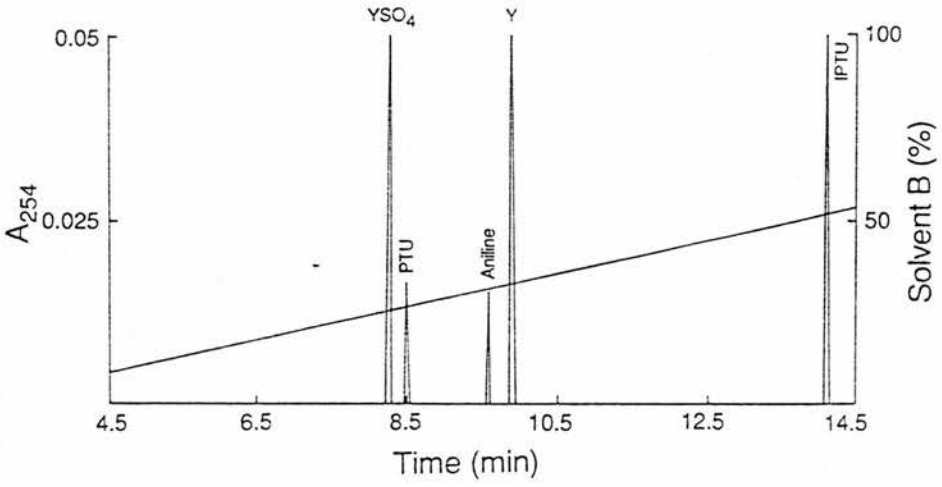


Fig. 3.33. Amino acid analysis of radiolabelled TRAMP alkaline hydrolysates

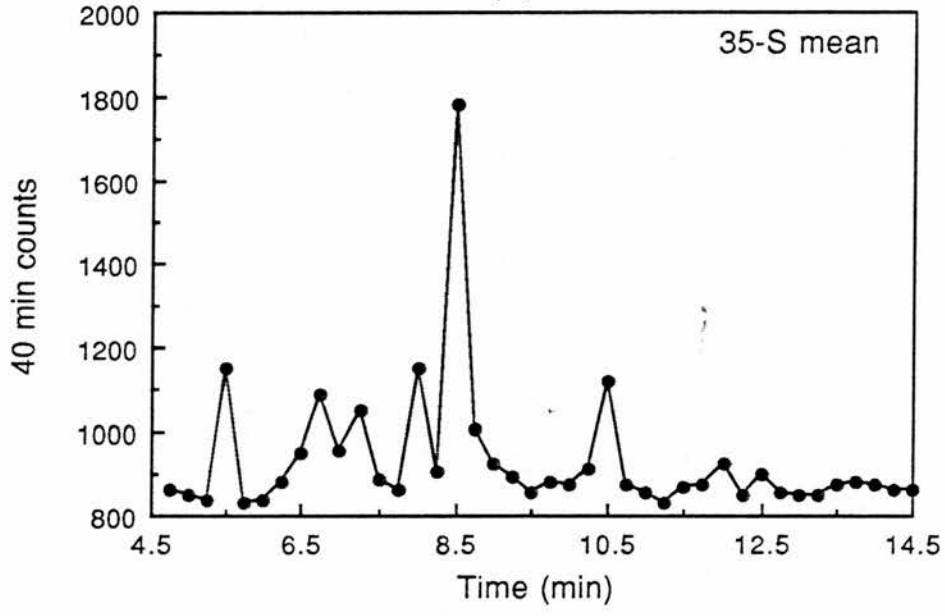
Sulphotyrosine and tyrosine standards and alkaline hydrolysates were separated on a PTC C-18 column (5 µm particle size; 2.1 mm x 200 mm) at a flow rate of 300 ml/min at 38°C.

(a) Sulphotyrosine, (b) tyrosine, (c) sulphotyrosine and tyrosine, (d) hydrolysate of ^{35}S-sulphate labelled TRAMP, (e) hydrolysate of [^{3}H]tyrosine labelled TRAMP.

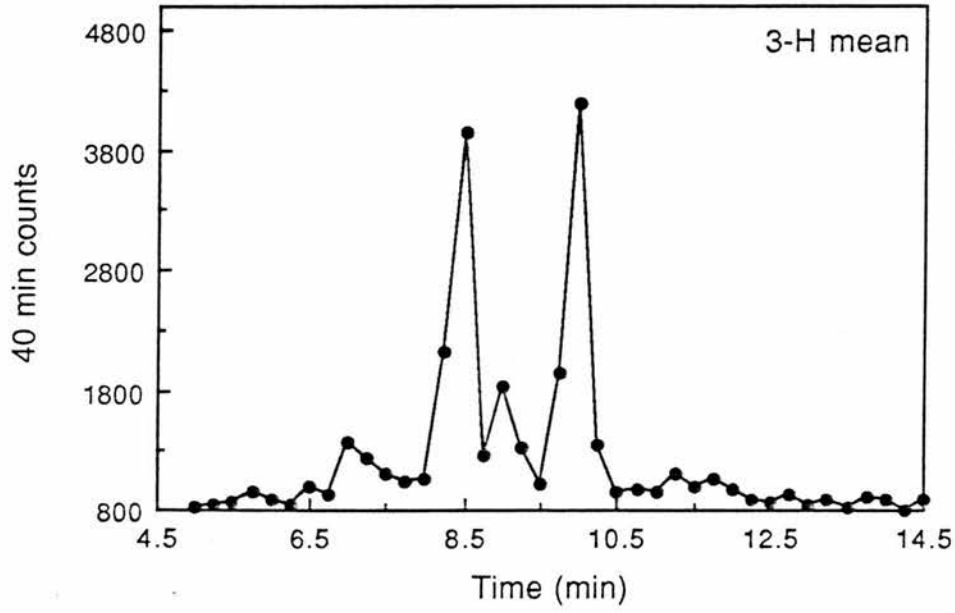
(c)


YSO4
Y
0.05
100
IPTU
A254
0.025
50
Solvent B (%)
PTU
Aniline
4.5
6.5
8.5
10.5
12.5
14.5
Time (min)

(d)


35-S mean
2000
1800
1600
1400
1200
1000
800
40 min counts
4.5
6.5
8.5
10.5
12.5
14.5
Time (min)

(e)


3-H mean
4800
3800
2800
1800
800
40 min counts
4.5
6.5
8.5
10.5
12.5
14.5
Time (min)

4. Discussion

4.1 Introduction

Although the cDNA derived amino acid sequence for lysyl oxidase has been found for several species, the location of the N-terminus and precise mass of the mature protein has not been clearly defined. This study shows that porcine lysyl oxidase has a mass of approximately 29K, which compares with the range 28-34K found by many workers by SDS-PAGE measurements (Table 1.2). From the results described here, I tentatively suggest that the N-terminus of porcine lysyl oxidase begins at residue D4 (Fig. 3.13.ii).

The present study has also shown that TRAMP is clearly distinct from lysyl oxidase and the complete amino acid sequence has been found. TRAMP has also been shown to contain sulphotyrosine residues which may account for the variant forms of this protein. Little is known about the function of TRAMP. It may be involved in mediating cell adhesion (Lewandowska *et al.*, 1991), and it has also been found, using lathyritic rat skin collagen, to accelerate fibril formation (MacBeath *et al.*, 1993). These results are now discussed in more detail below.

4.2 Lysyl oxidase

4.2.1 Amino acid sequence

4.2.1.1 Comparison with other sequences

The amino acid sequence data from porcine skin lysyl oxidase confirm that it corresponds to lysyl oxidase from rat aorta (Trackman *et al.*, 1990; Trackman *et al.*, 1991) human placenta (Hamalainen *et al.*, 1991) and chick aorta (Wu *et al.*, 1992). From the peptides sequenced so far (approximately 62% of the total sequence), porcine skin lysyl oxidase shows 98% identity with human lysyl oxidase (Hamalainen *et al.*, 1991) with differences at three residues S32 (G32), S145 (N145) and V150 (W150), where the human residues are shown in parentheses (Fig. 3.13.ii). A comparison of the amino acid sequence of the putative precursor region and

putative mature enzyme region from different species is shown in Table 4.1. The precursor region of lysyl oxidase (Fig. 3.13.i) shows a low level of inter-species sequence identity (Table 4.1a).

The protein encoded by the mouse *ras* recision gene shows 91% sequence identity with the precursor region of rat lysyl oxidase and 96% identity with the putative mature rat lysyl oxidase (Table 4.1; Kenyon *et al.*, 1991; Mariani *et al.*, 1992). The *ras* genes are oncogenes that were first discovered in viruses that caused rat sarcomas. These cell-derived viral genes may in some cases confer on the virus the ability to induce uncontrolled proliferation of infected cells and hence produce tumours in infected organisms. Therefore in addition to cross-linking, lysyl oxidase also appears to play a role in tumour supression, acting as a protectant against certain types of tumour. Specific variants of lysyl oxidase may be involved in tumour supressor activity.

4.2.1.2 N-terminus of lysyl oxidase

Prior to this study, the propeptide cleavage site of lysyl oxidase was unknown, as N-terminal sequencing of mature bovine aorta (Trackman *et al.*, 1990), human placenta (Hamalainen *et al.*, 1991) and porcine lysyl oxidase has been unsuccessful. A sequence SRRAA (residues 168-172; Fig. 3.13.i) in rat lysyl oxidase has been suggested as a possible cleavage site, the cleavage taking place between the first and second arginine i.e. between R169-R170 (Trackman *et al.*, 1991). However, this peptide sequence is not present in chick or human lysyl oxidase, although the residue R170 is conserved. A highly conserved region begins about 25 residues later in the C-terminal direction, and the data reported here suggest that the actual cleavage site may well be close to the beginning of this conserved region. Assuming cleavage occurs between residues G3-D4, the mature forms of lysyl oxidase (Fig. 3.13.ii), show a high level of sequence identity (Table 4.1b).

It is not neccessarily important that the N-terminal residue is conserved. Glycolytic enzymes, which are generally well conserved, show considerable variation in their N-terminal residues and some species have extended N-termini (reviewed by Fothergill-Gilmore and Michels, 1993). For example,

Table 4.1. Sequence identities in lysyl oxidase

(a) Amino acid identity in the putative lysyl oxidase precursor region

	Human	Rat	Mouse rrg	Chick
Human	100	72	74	34
Rat	72	100	91	30
Mouse rrg	74	91	100	35
Chick	34	30	35	100

(b) Amino acid identity in the putative mature lysyl oxidase enzyme

	Porcine	Human	Rat	Mouse rrg	Chick
Porcine	100	98	99	99	88
Human	98	100	94	96	91
Rat	99	94	100	97	93
Mouse rrg	99	96	97	100	93
Chick	88	91	93	93	100

The gaps and insertions in the different sequences were treated as residue differences.

chick muscle and human muscle pyruvate kinase have a N-terminal serine, whilst rat muscle pyruvate kinase has a N-terminal proline. Rat erythrocyte pyruvate kinase contains an N-terminal serine but also has an N-terminal extension of 42 amino acids.

From the evidence in this study, which comes from a variety of sources, I tentatively suggest that the mature lysyl oxidase enzyme starts at residue D4 (Fig. 3.13.ii). The reasoning behind this is as follows. Peptide VGD (residues 2, 3, 4; Fig. 3.13.ii), which follows a methionine residue, was not found after CNBr digestion of L3. This suggested that the N-terminus of mature lysyl oxidase begins somewhere on the C-terminal side of residue M1. (It must be emphasised that the numbering scheme is arbitrary and therefore M1 has nothing to do with its position in the mature sequence.) Further, endoproteinase-Asp-N sub-digestion of the blocked N-terminal CNBr fragment of L3 (Fig. 3.12) yielded several peptides which were sequenced. The peptide DDN (residues 14, 15, 16; Fig. 3.13.ii) was found. This suggested that the N-terminus of mature lysyl oxidase lies between residues V2 and T13. The Asp-N peptide DDP (residues 4, 5, 6) was not found initially when sequenced, but after exposure to 3M HCl vapour and re-sequencing, this peptide appeared with a low yield. This result indicates that the sequence DDP was present in the blocked N-terminal peptide, and hence the N-terminus was localised to V2, G3, or D4. Mass analysis of the blocked N-terminal Asp-N peptide (observed mass 1,272 +/- 1Da) was consistent with the actual mass of (1,261 +/- 1Da) of an N-terminal peptide beginning at D4 (Table 3.10). The mass difference (11 +/- 2Da) may be indicative of the mass of the blocking group. N-terminal Asp-N peptides beginning with V2 or G3 would have theoretical masses (excluding the blocking group) of 1,418 or 1,360 respectively. It should be noted that these comparisons of observed and theoretical masses assume identity of the human and porcine sequences, and also the absence of any post-translational modifications.

The nature of the blocking group is unknown. A large proportion (approximately 70%) of eukaryotic proteins are blocked at their N-termini. The main reason for N-terminal modification is not entirely clear but one function may be to protect proteins from proteolytic degradation by aminopeptidases. There is a strong preference for N-terminal blocking of amino acids which are

over-represented at the N-terminus i.e. where a residue is relatively common in the N-terminal region (Driessen *et al.*, 1985). There is no explanation for this at present. In the case of lysyl oxidase, aspartic acid is a relatively common residue. For example, D4 is adjacent to D5 in peptide DDN, where D is over-represented. Acetylation, the addition of an acetyl group (C_2H_3O; 43Da), accounts for 80-90% of blocked proteins (Aitken, 1990; Driessen *et al.*, 1985). The addition of a formyl group (CHO; 29Da) is also possible but less common. The N-formyl group is present on all bacterial proteins during biosynthesis, but is infrequently found in these proteins when isolated. Two methods described for unblocking proteins with N-formyl groups were tried on the intact lysyl oxidase without success (Section 2.7.2).

There is a preference for the acetylation of certain amino acids; 35-50% of acetylated proteins have acetyl-Ser; 27-33% have acetyl-Ala; 5-8% acetyl-Gly; 5-6% acetyl-Met; 5-6% acetyl-Thr; 1-3% acetyl-Asp; and 2-3% acetyl-Val (Arfin and Bradshaw, 1988; Driessen *et al.*, 1985). However, neither acetyl-Ser nor acetyl-Ala are likely to be the N-terminal residues of lysyl oxidase from the mass and sequence data presented here, which indicate an N-terminal aspartic acid. Proteins with acetylated-Asp N-termini are found in several contractile system and cytoskeleton proteins. These include actins from vertebrates, non-vertebrates and amphibia; and tropomyosin and troponin C from vertebrates (Driessen *et al.*, 1985). It has in fact been previously reported that the mature form of bovine aorta lysyl oxidase contains an aspartic acid at the N-terminus (Sullivan and Kagan, 1982; Kagan, 1986). In these studies, the N-terminal residue was identified by reacting lysyl oxidase with dansyl chloride at room temperature for 90 min, followed by acid hydrolysis. The dansyl-amino acid was then separated by thin layer chromatography and compared to a dansyl-amino acid standard mixture. However for a protein to react with dansyl chloride its N-terminus must be unblocked. As the N-terminus is known to be blocked, the validity of Sullivan and Kagan's (1982) observations is questionable. It is possible that limited proteolysis may have exposed an internal aspartate, e.g. D5, for subsequent dansylation.

Apart from acetyl- or formyl- groups at the N-terminus, the occurrence of cyclised glutamine residues (pyroglutamic acid) is one of the most common

post-translational modifications (Podell and Abraham, 1978). There are no glutamate or glutamine groups in the conserved region of the N-terminal mature form of lysyl oxidase (residues 1-13; Fig. 3.13.i), which suggests that such a blocking group is unlikely.

A wide range of proteins of different function and distribution are methylated at their N-termini (Stock *et al.*, 1987). Methylated N-termini may have one or more methyl blocking groups. N-terminal monomethylation (CH_2; 14Da) may occur on a methionine residue, e.g. *E. coli* ribosome protein L11, or an alanine residue, e.g. *E. coli* ribosome protein S11, or a phenylalanine residue, e.g. bacterial pili (Stock *et al.*, 1987). N-dimethylated and N-trimethylated eukaryotic proteins have proline or alanine at the N-terminus, which is followed by a Pro-Lys sequence. A consensus sequence has been identified for N-terminal dimethylated ($(CH_2)_2$; 28Da) and trimethylated ($(CH_2)_3$; 42Da) eukaryotic proteins, as is shown below:

$(CH_2)_2$-PPK e.g. cytochrome c557 from the protozoan *Crithidia oncopelti* (Pettigrew and Smith, 1977).

$(CH_2)_3$-APK e.g. rabbit myosin (Henry *et al.*, 1982).

Methylated amino acids are resistant to acid hydrolysis in 6M HCl at 110°C for 24h and an amino acid analyser can be used to identify the position of methylated residues. No unusual peaks were found in the amino acid analysis of lysyl oxidase.

A specific group of proteins have been shown to be N-terminally acylated with fatty acids (Trowler *et al.*, 1988). These contain myristic acid ($CH_3(CH_2)_{12}CO$; 211Da) covalently attached to glycine at the N-terminus, e.g. protein phosphatase inhibitor-1 from rabbit skeletal muscle (Aitken *et al.*, 1982). Since lysyl oxidase is believed to start at residue D4, such a large blocking group can be ruled out.

To overcome the problem of the blocked N-terminus, it might be possible to digest the N-terminal peptide with carboxypeptidase Y, which sequentially removes residues from the C-terminus, and to analyse the released amino acids on the amino acid analyser over a time course, to obtain the sequence. However this technique requires large amounts of peptide. It would be extremely useful to carry out C-terminal analysis of the N-terminal Asp-N peptide, DPP. Unfortunately, however this technique is still at

the development stage, requiring 2.5 nmols of sample due to low repetitive yields (Boyd *et al.*, 1992). Furthermore, certain residues cause problems, e.g. threonine and serine, which stop sequencing, and other residues are difficult to derivatise and detect by HPLC e.g. aspartic acid (P. Jackson, personal communication). Hence the blocking group of lysyl oxidase remains unclear.

4.2.1.3 Consensus sequences

Copper binding motifs have been found in four copper proteins, azurin (Ambler, 1971), plastocyanin (Milne *et al.*, 1974), pseudoazurin and amicyanin (Ambler and Tobari, 1985). The motif is shown below:

-H-(X)-C-X-X-H-X-X-M-

The copper binding ligands are the sulphur atom of the single cysteine residue and the sulphur atom in the methionine residue, together with the nitrogen atoms of the histidine imidazole groups (Hellinga and Richards, 1991).

Chick, human and rat lysyl oxidase share a common sequence HSCHQHYHSM (residues 124-133; Fig. 3.13.ii) which is similar to the above consensus sequence. This sequence is not found in tyrosinase, a copper-containing mono oxygenase, from *Streptomyces glancescens* (Huber *et al.*, 1985). However two copper-binding peptide sequences, HRRY (residues 214-217) and HHAY (residues 61-64) have been implicated in tyrosinase from *St. glaucescens* (Huber *et al.*, 1985). Lysyl oxidase contains similar sequences HRRF (residues 170-173; Fig. 3.13.ii) and HHAY (residues 240-243; Fig. 3.13.ii). The peptide HRRF (residues 170-173; Fig. 3.13.ii) is replaced by YRRY in chick lysyl oxidase but the peptide HHAY (residues 240-243; Fig. 3.13.ii) is entirely conserved in chick, human and rat lysyl oxidase. Interestingly, azurin (Ambler, 1971), plastocyanin (Milne *et al.*, 1974), pseudoazurin and amicyanin (Ambler and Tobari, 1985) do not contain the HRRY or HHAY sequences.

The copper binding motif found in azurin (Ambler, 1971), described above, is not found in the copper protein, galactose oxidase (McPherson *et al.*, 1992). The copper ion in galactose oxidase has five ligands in square pyrimidal coordination. The following residues form the square base, Y272,

H496, H581 and an acetate ion. The top of the pyramid is formed by Y495 (Ito *et al.*, 1991). There is also evidence for the involvement of a C-Y bridge and stacking interaction of these residues with a tryptophan.

4.2.1.4 Secondary structure prediction

The histogram in Fig. 4.1 shows the results of joint secondary structure prediction (Sawyer *et al.*, 1986) with the heavy horizontal bars indicating the regions of likely secondary structure of mature human lysyl oxidase (Hamalainen *et al.*, 1991) assuming that the N-terminal residue is D4 (Fig. 3.13.ii). This figure suggests that human lysyl oxidase contains 16% β-sheet and 18% α-helix.

The N-terminal region appears to consist of β-turns (residues 2-21, 28-33 and 33-40) which are proceeded by β-sheets (residues 40-45 and 54-64). There then follow α-helical regions (residues 70-90 and 92-96), a β-sheet region (residues 96-100), and a β-turn region (residues 95-111 and 113-118). The structure continues with a second split α-helix region (residues 113-136, 139-144, 147-153 and 157-160) followed by two β-turns (residues 161-169 and 181-186). In the C-termminal region, the structure alternates between β-sheet and β-turn as follows: β-sheet (residues 197-201), β-turn (residues 204-209), β-sheet (residues 208-216), β-turn (residues 222-228), β-sheet (residues 228-237) and β-turn (residues 243-248).

The secondary structure prediction described is based on seven separate prediction methods (Sawyer *et al.*, 1986). This is believed to be considerably more accurate than relying on individual prediction methods. However, it is interesting to compare the information in Fig. 4.1 with some of the separate predictions and also to look at the hydrophilicity of the enzyme. The prediction of β-turns, β-sheets and α-helices by the Chou-Fasman method (CF; Chou and Fasman, 1974) and Garnier, Osguthorpe and Robson method (GOR; Garnier *et al.*, 1978) are shown in Fig. 4.2. Analysis for regions of hydrophilicity by the Kyte-Doolittle method (KD; Kyte and Doolittle, 1982; Figs. 4.2 and 4.3) and by the GOR method (Fig. 4.4) indicate that lysyl oxidase is very hydrophilic, with small pockets of hydrophobicty (near residues 48, 82, 97, 157, 166, 202, 209-214, 218, and 232) and a hydrophobic C-terminus

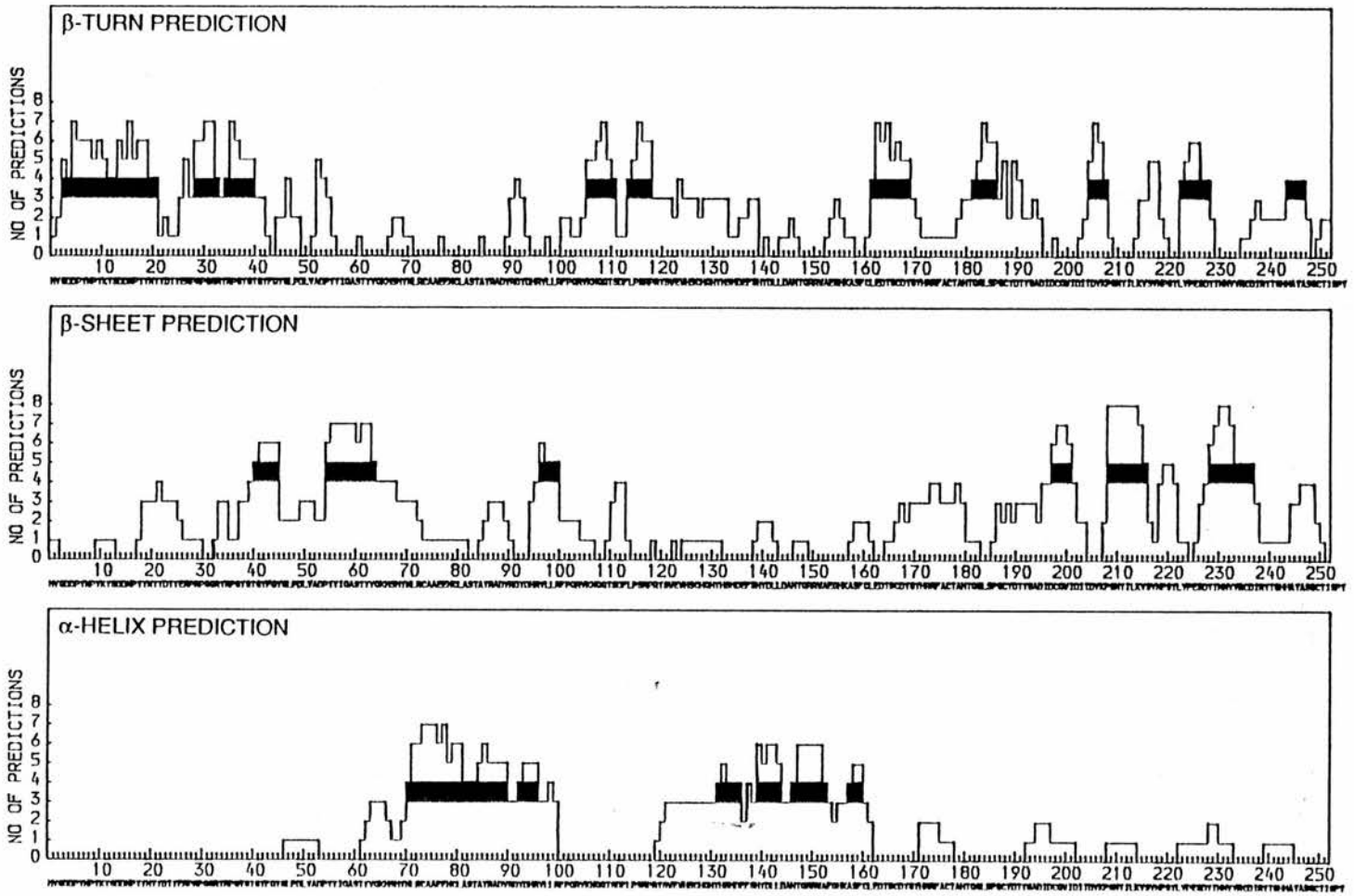


Fig. 4.1. Combined secondary structure predictions for β-turn (top), β-sheet (centre) and α-helix (bottom) for mature human lysyl oxidase (Hamalainen *et al.*, 1991)

It is assumed that the N-terminus of mature lysyl oxidase is at residue D4 (Fig. 3.13.ii). The vertical axis represents the number of separate predictions for each structural element and the horizontal axis gives the residue and residue number. The heavy horizontal bars are those regions that are deemed to be in the specification discussed in the text.

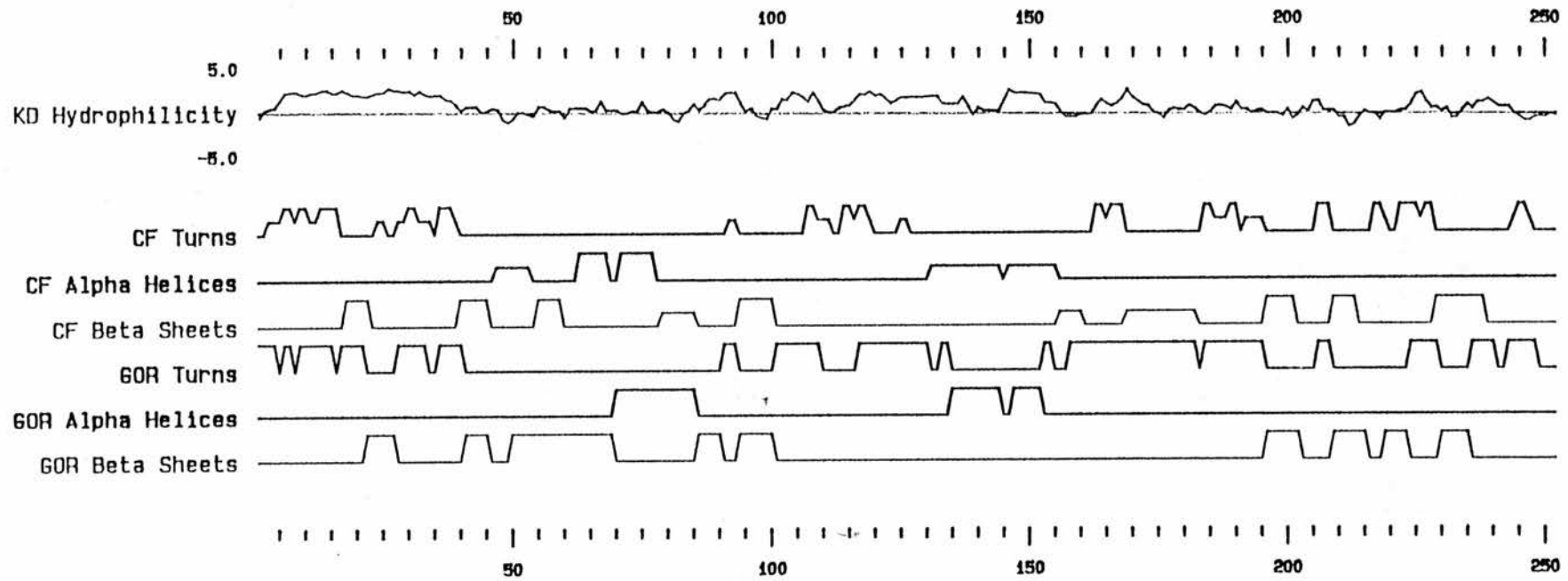


Fig. 4.2. Individual secondary structure predictions for hydrophilicity, β-turns, β-sheets and α-helices for mature human lysyl oxidase (Hamalainen *et al.*, 1991)

It is assumed that the N-terminus of mature lysyl oxidase is at residue D4 (Fig. 3.13.ii). The abbreviations used for the prediction methods are as follows: KD = Kyte-Doolitle, CF = Chou-Fasman and GOR = Garnier, Osguthorpe and Robson. The numbered scale (top and bottom) indicates the residue number.

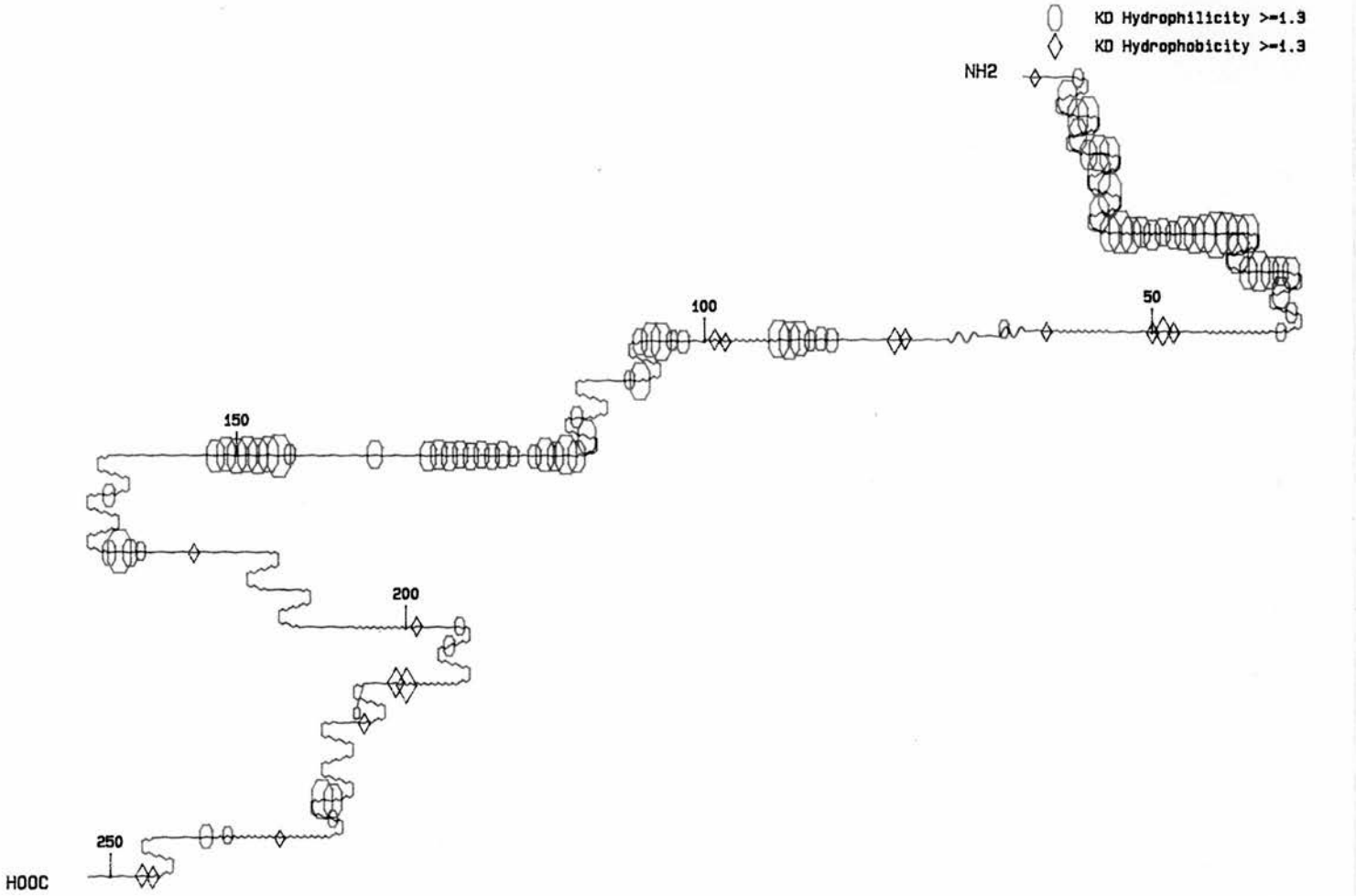


Fig. 4.3. Chou-Fasman prediction of the secondary structure of mature human lysyl oxidase (Hamalainen *et al.*, 1991)

It is assumed that the N-terminus of mature lysyl oxidase is at residue D4 (Fig. 3.13.ii). The patterns used in the plot are as follows: (〰) α-helices, (〰) β-sheets and (〰) β-turns.

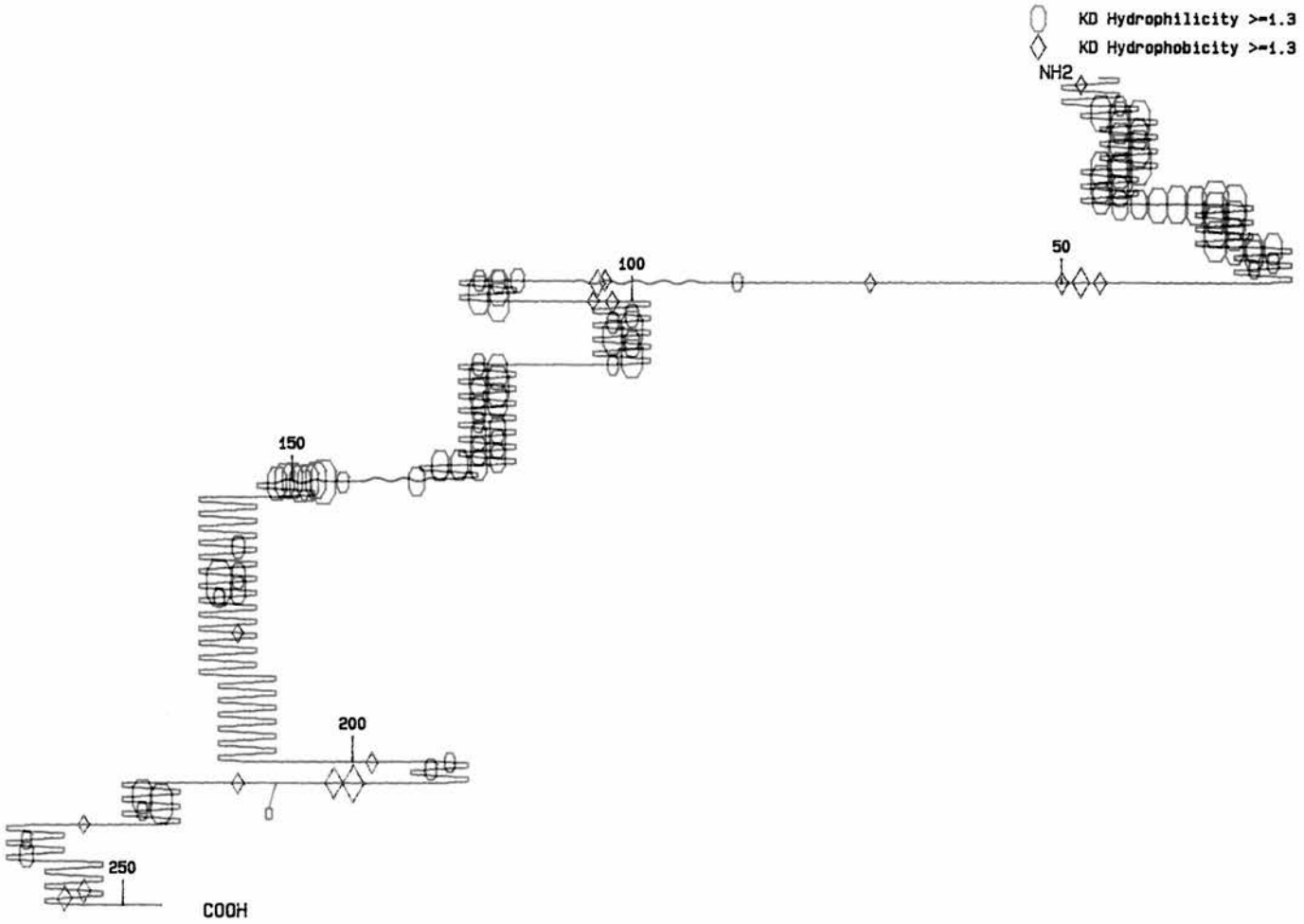


Fig. 4.4. Garnier, Osguthorpe and Robson prediction of the secondary structure of mature human lysyl oxidase (Hamalainen *et al.*, 1991)

It is assumed that the N-terminus of mature lysyl oxidase is at residue D4 (Fig. 3.13.ii). The patterns used in the plot are as follows: (~~~) α-helices, (~~~) β-sheets and (≋) β-turns.

(residues 245-252).

4.3 TRAMP

4.3.1 Amino acid sequence

The complete amino acid sequence of TRAMP has shown that this protein is not a degradation product of lysyl oxidase, as was originally suggested (Sullivan and Kagan, 1982; Kuivaniemi *et al.*, 1984; Burbelo *et al.*, 1986). The database search revealed that the TRAMP sequence showed 98% identity with the 22K dermatan sulphate proteoglycan-associated extracellular matrix protein from bovine skin (Neame *et al.*, 1989), differing at only four residues (Fig. 3.23).

4.3.2 Secondary structure prediction

The histogram in Fig. 4.5 shows the results of joint secondary structure prediction (Sawyer *et al.*, 1986) with the heavy horizontal bars indicating the regions of likely secondary structure of porcine TRAMP. This figure suggests that porcine TRAMP contains 13% β-sheet and 18% α-helix.

The N-terminal region appears to consist of β-turns (residues 2-8, 13-19, and 31-34) which then form a distinct β-sheet (residues 35-45) and then revert back to β-turns (residues 48-53, 59-64 and 67-70).

There then follows the first of four α-helices (residues 72-77), proceeded by a β-turn (residues 87-91), a β-sheet (residues 92-96) and the second α-helix (residues 102-111). The structure continues with a β-sheet (residues 111-117), β-turns (residues 120-124 and 130-135), followed by the third α-helix (residues 137-142). The β-turn (residues 143-147) forms a β-sheet (residues 153-158), and this is immediately followed by the fourth α-helix (residues 159-172) before entering into a final β-turn (residues 175-178).

As with lysyl oxidase, it is interesting to compare the information in Fig. 4.5 with some of the individual predictions and also to look at the

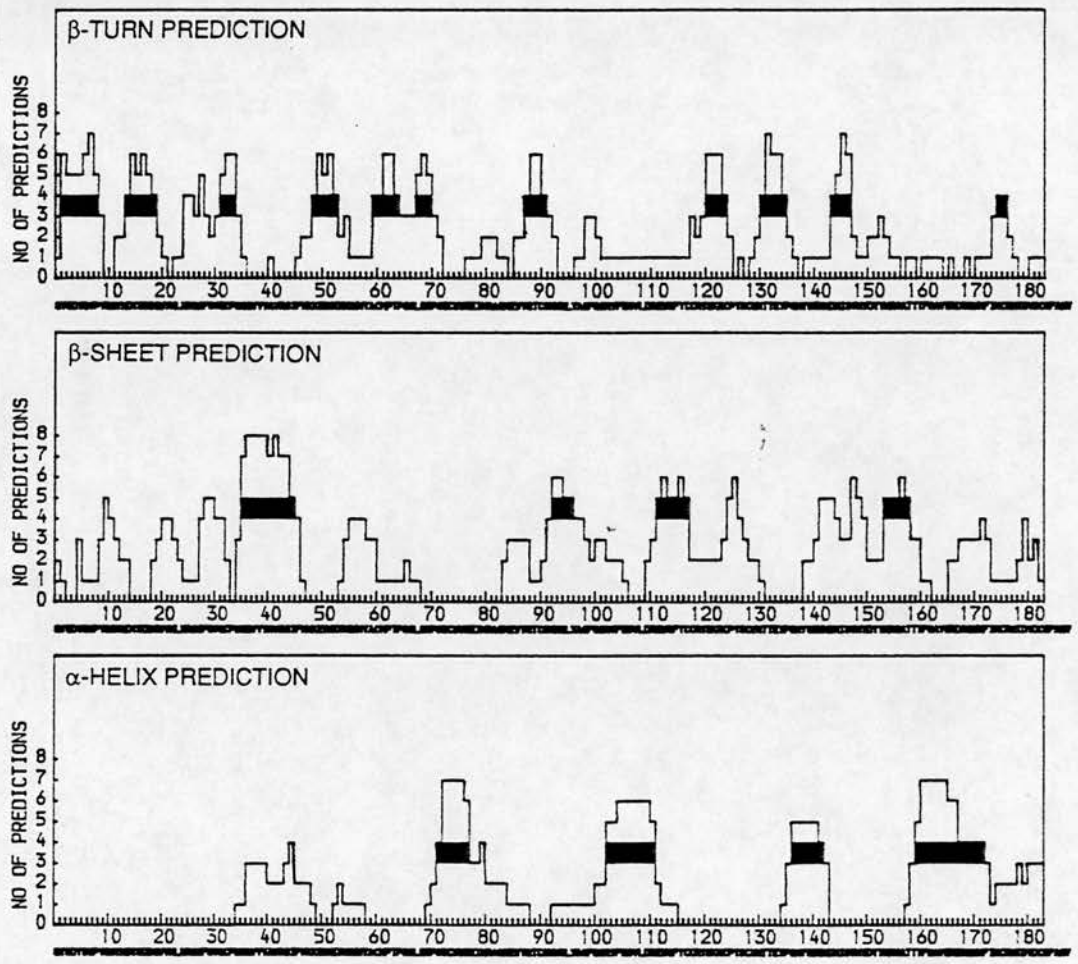


Fig. 4.5. Combined secondary structure predictions for β-turn (top), β-sheet (centre) and α-helix (bottom) for TRAMP

The vertical axis represents the number of separate predictions for each structural element and the horizontal axis gives the residue and residue number. The heavy horizontal bars are those regions that are deemed to be in the specification discussed in the text.

hydrophilicity of TRAMP. The prediction of β-turns, β-sheets and α-helices by the Chou-Fasman method (Chou and Fasman, 1974) and Garnier, Osguthorpe and Robson method (Garnier *et al.*, 1978) are shown in Fig. 4.6. Analysis for regions of hydrophilicity by the Kyte-Doolittle method (Kyte and Doolittle, 1982; Figs. 4.6 and 4.7) and by the GOR method (Fig. 4.8) indicate that TRAMP is very hydrophilic, with four regions of hydrophobicity (residues 35-43, 93-97, 155-158 and 167-172) and a hydrophobic C-terminus (residues 180-183).

4.3.3 Function of TRAMP

The function of TRAMP is not well understood. Interestingly, sequence analysis suggests it may be an amine oxidase. Recently, a consensus sequence for topa quinone has been identified at the active site of several copper amine oxidases (Mu *et al.*, 1992; Janes *et al.*, 1992). The motif is shown below:

- (Y/L/G/A) - N - **X** - (D/E) - (Y/N) -

where **X** is the tyrosine residue that is modified to topa quinone. The biogenesis of topa quinone is thought to involve the sequential conversion of tyrosine to dopa, to dopa quinone, to topa, and topa to topa quinone (Fig. 4.9; Mu *et al.*, 1992). The TRAMP sequence YNYDY (residues 144-148; Fig.3.23) shows identity with this motif, and to a tryptic digest peptide of pig kidney diamine oxidase (Janes *et al.*, 1992). (I am grateful for discussions with E. Forbes on this sequence motif).

TRAMP also contains a six-residue consensus sequence of DR(Q/E)W(N/Q/K)(Y/P) (Neame *et al.*, 1989; Fig. 3.24). It is possible that these repeating domains may have collagen binding activity and this could promote binding between collagen molecules in a D-staggered array, thereby accounting for the acceleration of collagen fibril formation by TRAMP recently observed by MacBeath *et al.* (1993).

It has been observed that TRAMP binds to collagen and accelerates fibril formation *in vitro* (MacBeath *et al.*, 1993). Monomeric collagen can self-associate into fibrils by incubation at 30°C at neutral pH. The growth of fibrils can be monitored by measurement of the solution turbidity (Na *et al.*,

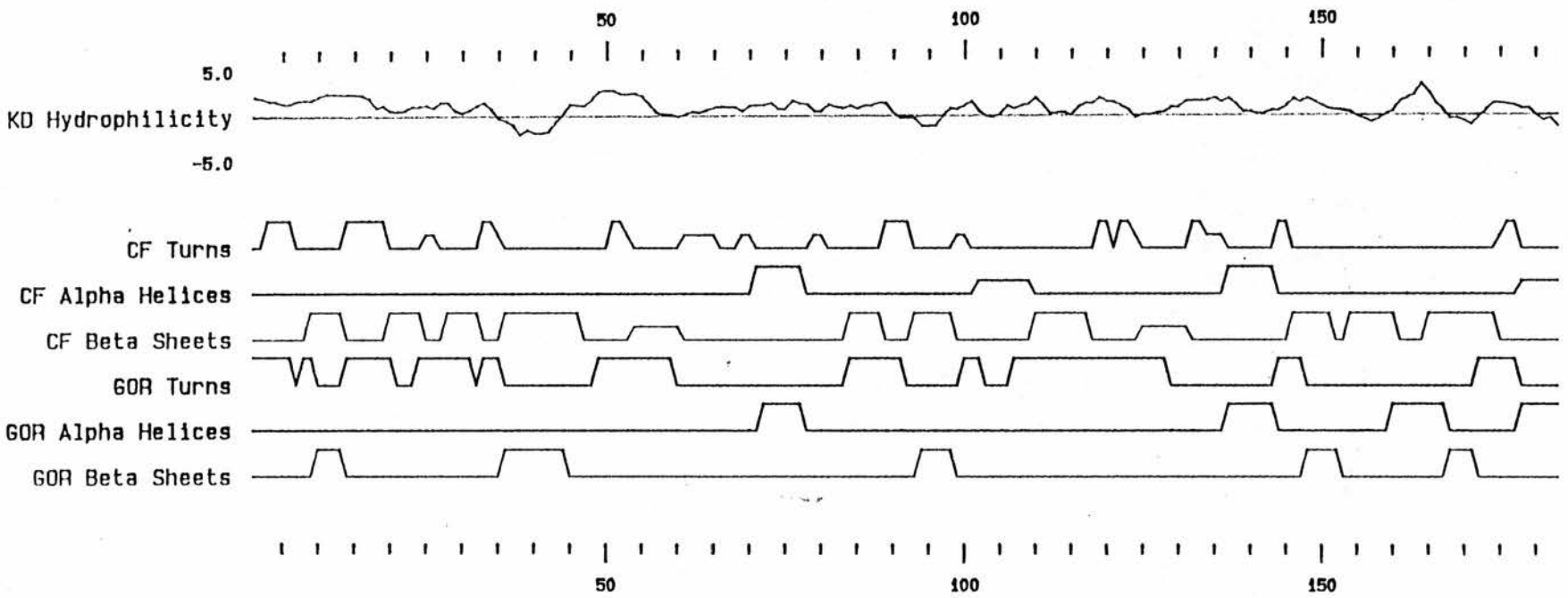


Fig. 4.6. Individual secondary structure predictions for hydrophilicity, β-turns, β-sheets and α-helices for TRAMP

The abbreviations used for the prediction methods are as follows: KD = Kyte-Doolitle, CF = Chou-Fasman and GOR = Garnier, Osguthorpe and Robson. The numbered scale (top and bottom) indicates the residue number.

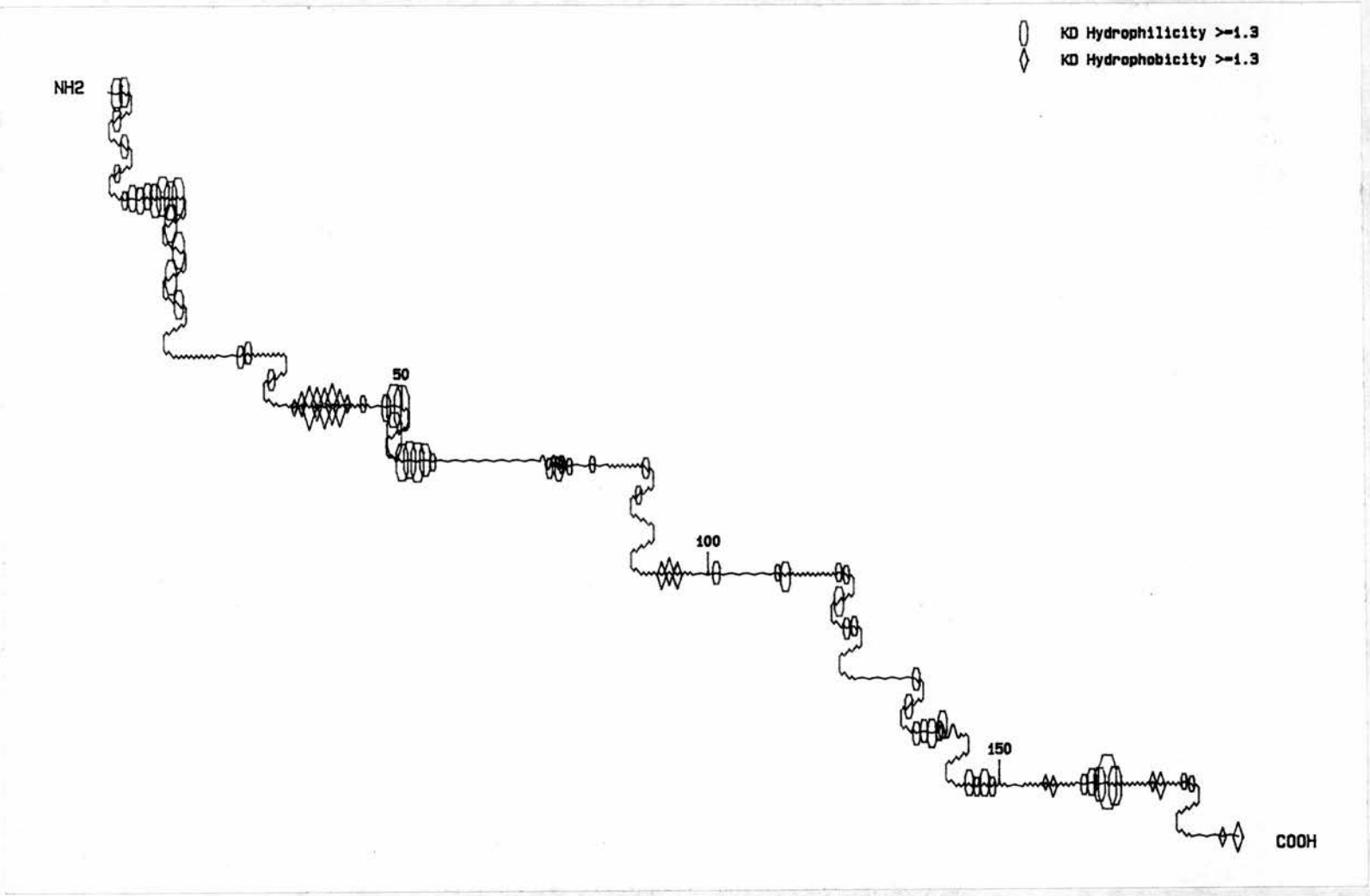


Fig. 4.7. Chou-Fasman prediction of the secondary structure of TRAMP

The patterns used in the plot are as follows: ( ) α-helices, ( ) β-sheets and ( ) β-turns.

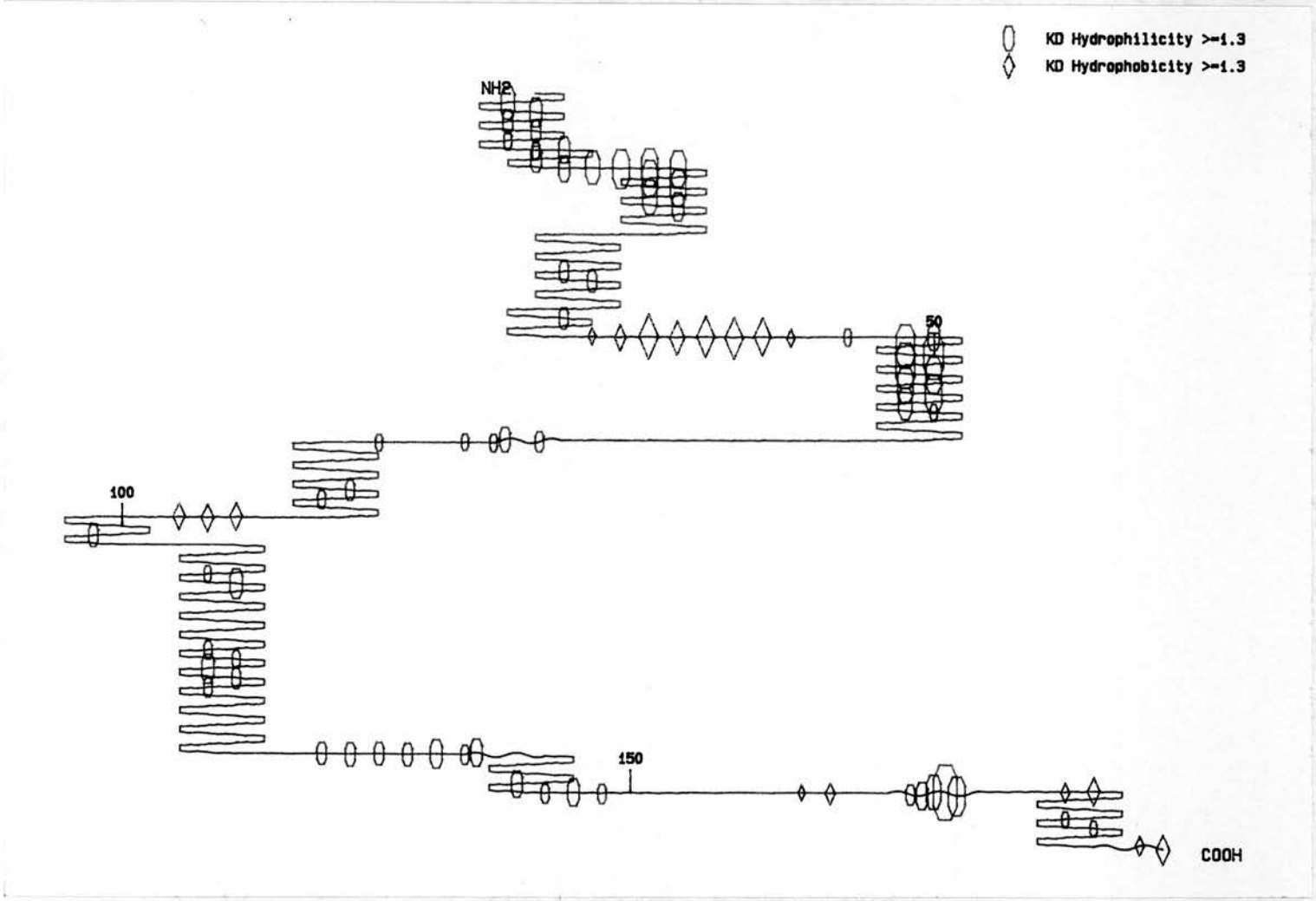


Fig. 4.8. Garnier, Osguthorpe and Robson prediction of the secondary structure of TRAMP

The patterns used in the plot are as follows: ( ) α-helices, ( ) β-sheets and ( ) β-turns.

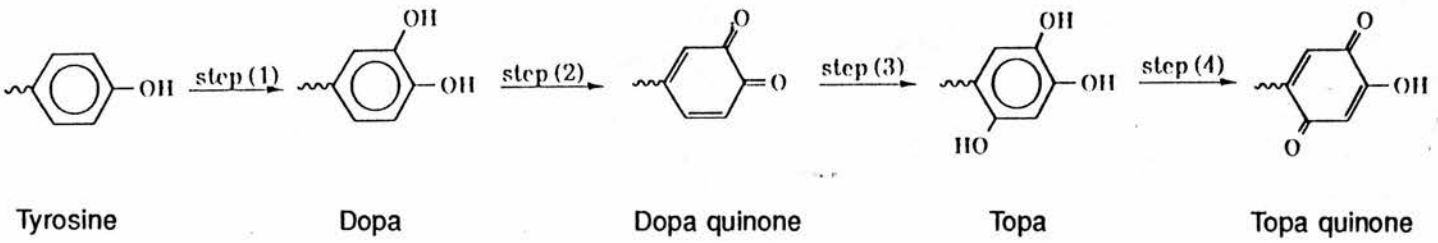


Fig. 4.9. Postulated pathway for the biogenesis of topa quinone from tyrosine

The reaction has been shown in four steps involving tyrosine hydroxylation to dopa, dopa oxidation to dopa quinone, dopaquinone hydration to topa and topa oxidation to topa quinone (Mu *et al.*, 1992).

1986). TRAMP may stimulate collagen synthesis by aggregating collagen monomers in preparation to cross-linking by lysyl oxidase. TRAMP may also work in conjunction with dermatan sulphate proteoglycan with which the bovine form of the protein has been shown to interact (Choi *et al.*, 1989; Neame *et al.*, 1989; Lewandowska *et al.*, 1991). The dermatan sulphate proteoglycan, decorin is known to inhibit fibril formation (Brown and Vogel, 1989; L. Rosenberg, personal communication). After fibril formation decorin becomes displaced to the outside of the collagen fibril (Scott, 1992). It is possible therefore that TRAMP acts antagonistically to decorin as part of a complex regulatory mechanism for the control of collagen fibril formation. Cell adhesion to bovine TRAMP (Lewandowska *et al.*, 1991) suggests a further role for this protein in cellular control of fibril deposition.

4.4 Comparison of lysyl oxidase and TRAMP

4.4.1 Amino acid sequence

Neither lysyl oxidase nor TRAMP have an RGD cell binding motif (Ruoslahti and Pierschbacher, 1986) as found in fibronectin, laminin and other glycoproteins. However, TRAMP contains a RGAT sequence (residues 151-154) which may be a cell-binding domain that can be recognised by members of the integrin family (Lewandowska *et al.*, 1991).

A feature that is common to both lysyl oxidase and TRAMP is the presence of very few lysines, i.e. four or five in lysyl oxidase and four in TRAMP. A further observation is that both proteins have two distinct tyrosine-rich domains (Fig. 4.10), one near the N-terminus (residues 4 to 25 in lysyl oxidase (Fig. 3.13.ii), residues 1 to 5 in TRAMP (Fig. 3.23) and another approximately 65% towards the lysyl oxidase C-terminus (residues 167 to173, Fig. 3.13.ii), and 80% towards the TRAMP C-terminus (residues 144 to 149, Fig. 3.23). Comparison of the entire sequences of lysyl oxidase and TRAMP (Fig. 4.11) showed 22% identity when aligned for maximum homology using the Wisconsin GCG program "Bestfit".

The sequence YDYYMRGATT in TRAMP (residues 146-155; Fig. 3.23)

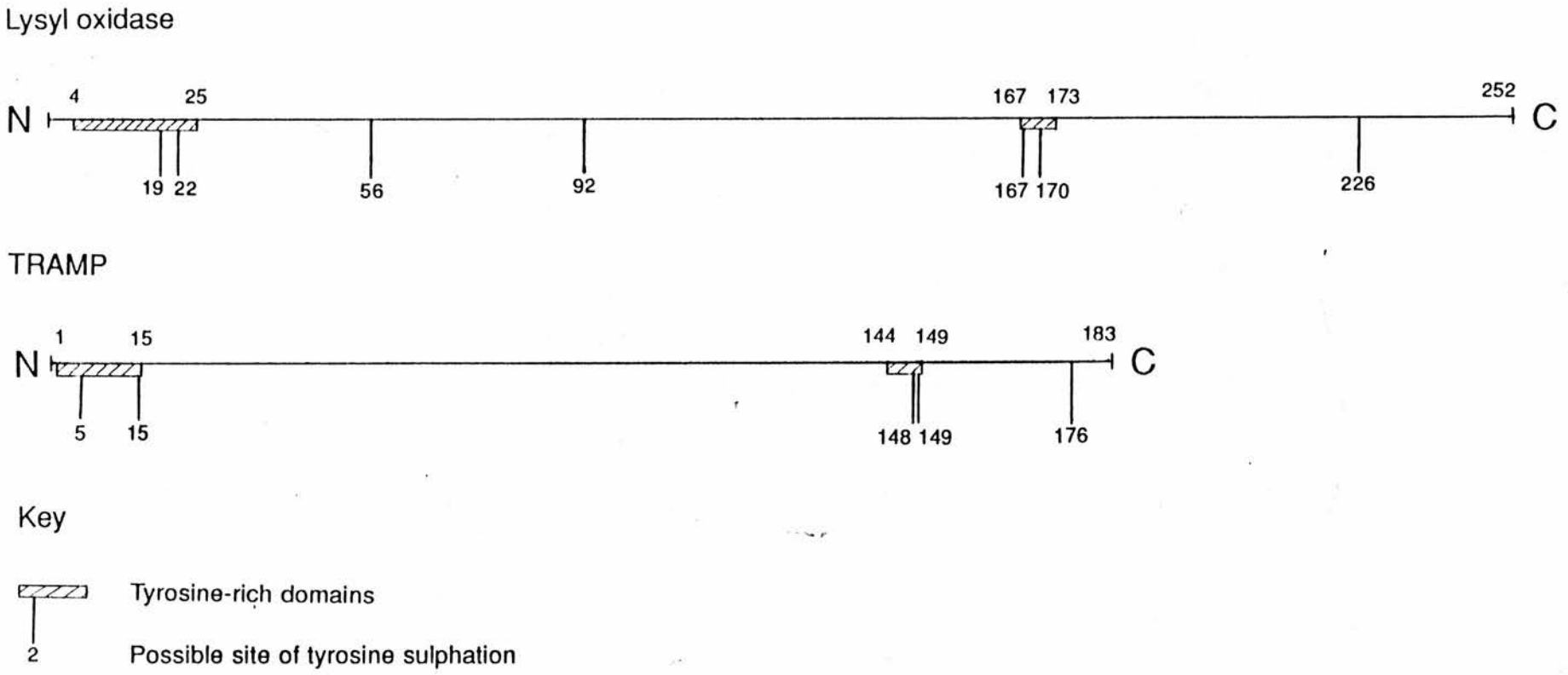


Fig. 4.10. Comparison of tyrosine rich domains and possible tyrosine sulphation sites in lysyl oxidase and TRAMP

The numbers shown indicate the residue number (Figs. 3.13.ii and 3.24).

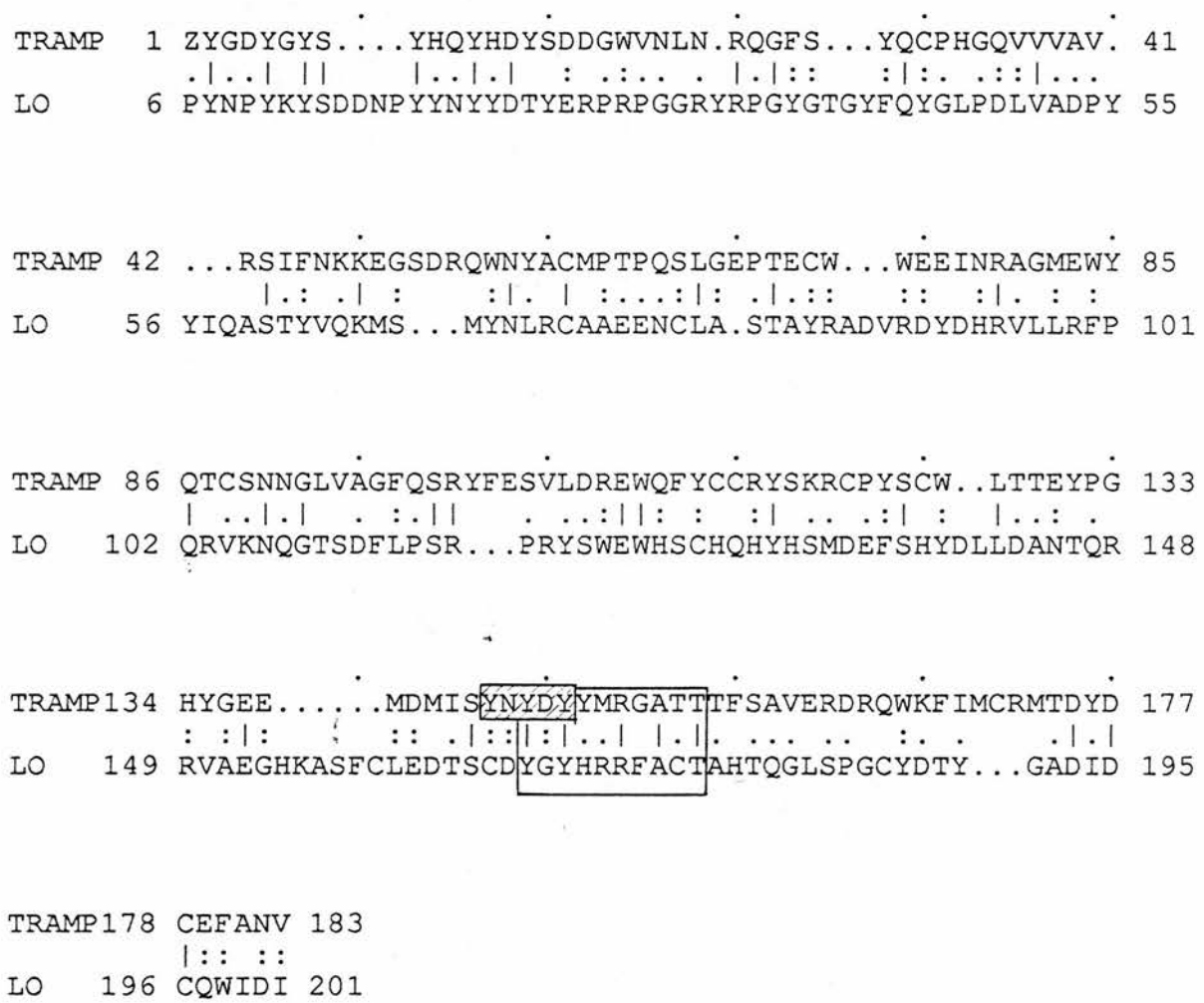


Fig. 4.11. Comparison of amino acid sequences of bovine TRAMP (Neame *et al.*, 1989) and human lysyl oxidase (LO; Hamalainen *et al.*, 1991)

The alignment symbols used are: (I) identity, (:) strongly positively conserved substitution, (.) weakly positively conserved substitution. The shaded box indicates the location of the consensus sequence for topa quinone. The open box indicates a region of homology to certain conserved copper-binding sequences (Trackman *et al.*, 1990).

is similar to the lysyl oxidase sequence YGYHRRFACT (residues 167-176; Fig. 3.13.ii) which resembles certain conserved copper-binding sequences (Trackman *et al.*, 1990; Fig. 4.11). This stretch of sequence overlaps with the suggested site of topa quinone in TRAMP (Section 4.3.1). TRAMP does not have a sequence similar to the other copper-binding consensus sequence in lysyl oxidase, HHAY (residues 240-243; Fig. 3.13.ii), nor does it have a HSCHQHYHSM (residues 124-133; Fig. 3.13.ii) copper binding motif, although it does have a sequence GMEWYQTCSN (residues 81-90; Fig. 3.23) which shows some similarity in reverse. However, the histidine residues, crucial for copper binding, are absent in this sequence.

The sequence SCDYGYH in L3 (residues 164 to 170, Fig. 3.13.ii) shows some similiarity to the sequence SYNYDYY in T3 (residues 143 to 149, Fig. 3.23), as shown in Fig. 4.11. This may suggest some similarity in function of the two proteins. Surprisingly, lysyl oxidase does not contain a topa quinone consensus sequence, though the exact nature of the organic quinone-type co-factor in lysyl oxidase is yet to be established (Kagan and Trackman, 1991).

4.4.2 Secondary structure prediction

The N-termini of lysyl oxidase and TRAMP each consist of a β-turn region (Figs. 4.1 and 4.5). Lysyl oxidase contains 16% β-sheet compared to 13% β-sheet in TRAMP. However, both lysyl oxidase and TRAMP each contain 18% α-helix. There are two split α-heliclal regions in lysyl oxidase and four distinct α-helices in TRAMP. Both proteins are very hydrophilic with each containing a highly hydrophilic N-terminus and a hydrophobic C-terminus (Figs. 4.3, 4.4, 4.7 and 4.8).

4.4.3 Significance of variant forms of lysyl oxidase and TRAMP

It has not been clear why both lysyl oxidase and TRAMP should exist in several variant forms. The presence of multiple, ionic variants of lysyl oxidase has also been observed in enzyme preparations from chick cartilage (Stassen, 1976), bovine aorta (Sullivan and Kagan, 1982) and human

placenta (Kuivaniemi *et al.*, 1984). Only one gene for lysyl oxidase has been found in rat genomic DNA (Trackman *et al.*, 1990), so post-translations are a likely cause of the variation. A possible modification considered was carbamylation at lysine residues while proteins are in 6M urea (Means and Feeney, 1971). In TRAMP, the decreasing isoelectric points (pI) for variants T1 to T5 are consistent with an increasing extent of carbamylation since, from complete amino acid sequence data (Fig. 3.23), the theoretical pI's would be 4.53, 4.44, 4.36, 4.28 and 4.20 (with pyroglutamate at the N-terminus and 0, 1, 2, 3 or 4 lysines carbamylated respectively; Table 3.3). In lysyl oxidase, the decreasing pI values for variants L1 to L4 are also consistent with an increasing extent of carbamylation. However, the observations from mass spectrometry are inconsistent with carbamylation. The additional mass for each lysine residue carbamylated should be 43Da, while the average mass increment between consecutive lysyl oxdase and TRAMP variants was found to be 65 and 59Da, respectively (Tables 3.6 and 3.16; Figs. 3.10 and 3.27). If carbamylation was the basis for variation, conversion to more acidic form would be expected when separated variants are further exposed to 6M urea. Prolonged incubations of purified TRAMP variants in 6M urea showed no evidence for such conversion. Similar observations were also reported by Kagan *et al.* (1979) on purified lysyl oxidase variants.

The experimental data reported here demonstrate the presence of tyrosine sulphation in TRAMP (Cronshaw *et al.*, 1993). Incubation of variant T3 with sulphatase showed that removal of sulphate changed the elution position of this protein on a Mono Q column. The appearance of three new peaks on the Mono Q column following sulphatase treatment of T3 suggests that up to three sulphate groups were removed, resulting in a corresponding increase in pI and hence earlier elution from the column. The elution positions of the two peaks to the left of T3 (following sulphatase treatment) correspond to those of the T1 and T2 variants (without sulphatase treatment).

The incremental mass difference due to tyrosine sulphation should be 79Da, which is greater than the mass increment found using the Lasermat mass analyser (Tables 3.6 and 3.16; Figs. 3.10 and 3.27). There is no clear explanation for this at present. More accurate mass analyses could be obtained using an electrospray mass spectrometer, if the proteins were

suitable for this form of analysis (Edmonds and Smith, 1990).

There are five empirical rules for predicting the location of sites of tyrosine sulphation (Hortin *et al.*, 1986). There must be an acidic residue at position -1 or -2, and at least three acidic residues within five residues (positions -5 to +5) of the tyrosine residue. No more than one basic residue must be within five residues of the tyrosine and no more than three hydrophobic residues (Ile, Leu, Phe and Val) also within five residues of the tyrosine. No cysteine residues must be within 15 residues of the tyrosine (Huttner, 1984). Sequence analysis indicates five possible tyrosine sulphation sites in TRAMP (residues 5, 15, 148, 149, and 176; Fig. 4.10). The difference between the observed and calculated molecular mass for variant T1 (approximately 130Da) is consistent with sulphation of two tyrosine residues (i.e. additional mass 2 x 79Da), within experimental error. The presence of two sulphated tyrosines would be expected to lower the isoelectric point of each TRAMP variant by about 0.17 pH unit, which corresponds to the difference between the observed and calculated pI's.

Tyrosine sulphation is a widespread post-translational modification in extracellular matrix proteins, e.g. nidogen/entactin (Paulson *et al.*, 1985), procollagen V (Fessler *et al.*, 1986), procollagen III (Jukkola *et al.*, 1986), bone sialoprotein II (Ecarot-Charrier *et al.*, 1989; Midura *et al.*, 1990), fibronectin (Liu and Lipman, 1985), dermatan sulphate proteoglycan (Huttner, 1988) and fibromodulin (Antonsson *et al.*, 1991). The site of tyrosine sulphation in fibromodulin is in an N-terminal tyrosine-rich region in which tyrosine is every second or third residue (Antonsson *et al.*, 1991). This feature is also present in TRAMP (Neame *et al.*, 1989), though the sequences are not homologous. Multiple variants of fibromodulin have also been observed but the effect of sulphatase on these variants has not been studied (R.M. Lauder, I.A. Nieduszynski and T.N. Huckerby, personal communication). The post-translational modification of tyrosine takes place in the trans-Golgi, by a tyrosylprotein sulphotransferase that uses 3'-phosphoadenosine 5'-phosphosulphate as a sulphate donor. The biological role of tyrosine sulphation in extracellular proteins is generally not clear, though desulphation of fibronectin affects binding affinities to several ligands (Suiko and Liu, 1988).

Tyrosine sulphation also occurs in plasma proteins, e.g. complement protein C4 and several peptide hormones, e.g. gastrin and cholecystokinin (Brand *et al.*, 1984). The activation of complement C4 is modulated by sulphation (Hortin *et al.*, 1989). This modification increases its haemolytic activity and its proteolysis by complement factor C1s. The hormone cholecystokinin (CCK) regulates pancreatic enzyme secretion. Sulphation of CCK increases its ability to stimulate secretion 100 fold (Brand *et al.*, 1984). Sulphation of gastrin makes the peptide more potent in pancreatic secretion (Brand *et al.*, 1984).

In comparison to TRAMP, lysyl oxidase does not stain with Alcian Blue (Cronshaw *et al.*, 1993). This suggests that lysyl oxidase is not sulphated. However, sequence analysis of lysyl oxidase indicates seven possible sites of tyrosine sulphation (residues 19, 22, 56, 92, 167, 170 and 226; Fig. 4.10).

4.5 Future work

4.5.1 Lysyl oxidase

Future work on porcine lysyl oxidase should concentrate on determining the complete sequence of the protein. As the next stage lysyl oxidase should be incubated with 5% formic acid at 60°C for 16h to try to cleave the Asp-Pro bonds at residues D6-P7 and D53-P54 (Fig. 3.13ii; Ambler *et al.*, 1984). This should also help in determining the N-terminus. It is also necessary to address the question of the variants of lysyl oxidase. The L3 variant of lysyl oxidase should be treated with sulphatase and run on the Mono Q colunn to see if there is a shift in elution position, as observed for the T3 variant of TRAMP (Fig. 3.29). Tyrosine sulphation however may not be the answer as lysyl oxidase did not stain with Alcian Blue (Cronshaw *et al.*, 1993). An antibody to lysyl oxidase should be prepared and used to purify [^{3}H]tyrosine and ^{35}S-sulphate labelled lysyl oxidase from cultured fibroblasts in similar experiments to those described for TRAMP should a change in elution position on a Mono Q column be observed after treatment with sulphatase.

4.5.2 TRAMP

Possible future work on TRAMP can concentrate on either its biosynthesis or function. Using degenerate primers based on the amino acid sequence data, it may be possible to identify a precursor form of TRAMP by polymerase chain reaction (PCR) amplification of cDNA libraries. The use of chlorate as an inhibitor of tyrosine sulphation may facilitate studies of sulphated and unsulphated forms of TRAMP (Baeuerle and Huttner, 1986). To further study the function of TRAMP immunohistochemistry could be used to reveal the tissue distribution of this newly discovered protein.

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Chapter 6 Appendix

6. Appendix

Poster presentations:

Cronshaw, A.D., Fothergill-Gilmore, L.A., Shackleton, D.R., Hulmes, D.J.S. and Tennant, B. (1990) *Amino acid sequence studies on lysyl oxidase.* XIIth. Federation of European Connective Tissue Societies (FECTS) Transactions, Bialystock, Poland.

Cronshaw, A.D., Fothergill-Gilmore, L.A., Hulmes, D.J.S.,Keane, A. and Shackleton, D.R. (1991) *Lasermat mass analysis of lysyl oxidase and Tyrosine Rich Acidic Matrix Protein (TRAMP).* XIIIth. FECTS Transactions, Davos, Switzerland.

Publication:

Cronshaw, A.D., MacBeath, J.R.E., Shackleton, D.R., Collins, J.F., Fothergill-Gilmore, L.A. and Hulmes, D.J.S. (1993) *TRAMP (Tyrosine Rich Acidic Matrix Protein), a protein that co-purifies with lysyl oxidase from porcine skin. Identification of TRAMP as the dermatan sulphate proteoglycan associated 22K extracellular matrix protein.* Matrix **1 3**: 255-266.

Three papers in press:

1. The complete amino acid sequence of TRAMP.
2. The N-terminal sequence of lysyl oxidase.
3. The biosynthesis, tissue distribution and tyrosine sulphation of TRAMP.

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TRAMP (Tyrosine Rich Acidic Matrix Protein), a Protein that Co-purifies with Lysyl Oxidase from Porcine Skin

Identification of TRAMP as the dermatan sulphate proteoglycan-associated 22K extracellular matrix protein

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Abstract

A protein (M_r 24 K) that co-purifies with porcine skin lysyl oxidase (M_r 34 K) has been isolated and characterised. Five variants of the 24 K protein were identified by Mono Q ion-exchange FPLC, as were four variants of lysyl oxidase. Amino acid analysis and partial sequencing revealed near identity of a 36-residue CNBr peptide from porcine skin lysyl oxidase to corresponding regions of the putative lysyl oxidase precursor derived from rat and human cDNA. The 24 K protein was found to be unrelated to lysyl oxidase, but comparison with a protein sequence database showed it to be the same as a recently described protein from bovine skin that is associated with dermatan sulphate proteoglycans. The 24 K protein is relatively rich in tyrosine, and isoelectric focussing shows it to be acidic, with pI's in the range 4.1 to 4.4. In view of these properties, we propose the name TRAMP (Tyrosine Rich Acidic Matrix Protein) to identify this protein. Though TRAMP appears not to be glycosylated, several experiments indicate the presence of sulphotyrosine residues. When assayed using an elastin substrate, the activity of lysyl oxidase is unaffected by TRAMP.

Key words: lysyl oxidase, protein structure, skin, TRAMP.

Introduction

Covalent cross-linking in collagens and elastin is essential for connective tissue structure and function (Kagan, 1986; Kagan and Trackman, 1991). The enzyme that initiates cross-linking, lysyl oxidase (EC 1.4.3.13), has been purified from several sources, including chick cartilage (Stassen, 1976); bovine aorta (Kagan et al., 1979; Sullivan and Kagan, 1982), ligament (Jordan et al., 1977) and lung (Cronlund and Kagan, 1986); human placenta (Kuivaniemi et al., 1984) and umbilical cord (Burbelo et al., 1986); porcine skin (Shackleton and Hulmes, 1990a) and rat skin (Romero-Chapman et al., 1991). Amino acid sequences of rat (Trackman et al., 1990; Trackman et al., 1991) and human (Hamalainen et al., 1991) lysyl oxidase precursors have recently been deduced from cDNA analysis. In many preparations of enzyme (Sullivan and Kagan, 1982; Kuivaniemi et al., 1984; Burbelo et al., 1986; Shackleton and Hulmes, 1990a), lysyl oxidase (30–34 K) is contaminated with one or more low molecular mass proteins (22–24 K). These contaminants have previously been suggested to be either degradation products of lysyl oxidase (Sullivan and Kagan, 1982; Kuivaniemi et al., 1984; Burbelo et al., 1986) or unrelated proteins (Kuivaniemi et al., 1984).

We have recently identified a 24 K protein in prepara-

tions of porcine skin lysyl oxidase (34 K) when purified by specific interaction with Sephacryl S-200 (Shackleton and Hulmes, 1990a). Here we describe an improved purification procedure and the identification of multiple variants of both the 24 K protein and lysyl oxidase. The 24 K protein is unrelated to lysyl oxidase. It is acidic and rich in tyrosine, so we propose the name TRAMP (Tyrosine Rich Acidic Matrix Protein) to identify this protein. By amino acid sequencing, we show that TRAMP is the same as a recently described 22 K protein from bovine skin that is associated with dermatan sulphate proteoglycans (Neame et al., 1989; Lewandowska et al., 1991).

Materials and Methods

Reagents

Unless stated otherwise, all reagents were obtained from BDH (Merck Ltd., Poole, Dorset, UK) or Sigma (Poole, Dorset, UK). Chromatography media (Sephacryl S-400, Sephadex G25, DEAE-Sepharose Fast Flow, CM-Sepharose Fast Flow) and reagents for isoelecric focussing (Ampholines, protein pI standards) were from Pharmacia, Milton Keynes, Bucks, UK. Reagents for the [^{3}H]elastin lysyl oxidase assay were as described (Shackleton and Hulmes, 1990a, b). Ultrafree-MC ultrafiltration units (10000 NMWL, PLGC membranes) were from Millipore (UK) Ltd, Watford, Herts, UK. Centricon C-10 microconcentrators (10000 MW cut-off) were from Amicon Ltd, Stonehouse, Glos, UK. Amino acid standards solvents and all reagents for HPLC, amino acid analysis and sequencing were from Applied Biosystems (UK), Warrington, Lancs, UK. 4-vinyl pyridine was from Aldrich (Gillingham, Dorset, UK). Neuraminidase *(Vibrio cholerae)*, O-glycosidase (BSA free), arylsulfatase *(Helix pomatia)* and β-glucuronidase *(E. coli)* were from Boehringer Mannheim, Lewes, East Sussex, UK. Fetuin, endoglycosidase F/N-glycosidase F, sulphatases (type V (limpet) and type VII (abalone)) were from Sigma.

Purification of lysyl oxidase and TRAMP

Piglet skin lysyl oxidase was prepared using a procedure similar to that described previously (Shackleton and Hulmes, 1990a), but with the following modifications to improve separation, increase capacity and reduce the time required. Typically skins from 15 stillborn piglets were used (approximately 1 to 2 kg wet weight). After discarding the initial extracts in phosphate buffered saline (0.1 M sodium phosphate pH 7.8, 0.15 M NaCl) and phosphate buffer (PB; 10 mM sodium phosphate, pH 7.8), extracts in 6 M PBU (PB containing 6 M urea) were pooled and filtered through Whatman No. 113V and Whatman No. 3 filters. (To remove cyanate, all urea containing buffers were prepared from fresh stock solutions of 8 M urea that were deionised with Bio Rad AG 501-X8 mixed bed resin immed
ately before use.) Prior to anion-exchange chromatogra
phy, filtered urea extracts (approximately 2 litres) wer
diluted with PB to 2 M PBU and then passed through
500 ml column (5 cm × 25 cm) of CM-Sepharose Fas
Flow, previously equilibrated in 2 M PBU, at a flow rate c
30 ml/min. The flow-through solution was then immed
ately applied to a 500 ml column (5 cm × 25 cm) of DEAE
Sepharose Fast Flow, previously equilibrated in 2 M PBU
at a flow rate of 30 ml/min. The DEAE-Sepharose colum
was then washed with two column volumes of 2 M PBU
followed by two column volumes of PB, and then most c
the bound proteins (but not lysyl oxidase or TRAMP) wer
eluted with PB containing 0.3 M NaCl. To release boun
proteins containing lysyl oxidase activity but relativel
uncontaminated with TRAMP, the column was eluted, a
30 ml/min, with 3 M PBU containing 0.3 M NaCl. A subse
quent elution with 6 M PBU containing 0.5 M NaC
released predominantly TRAMP. Lysyl oxidase an
TRAMP were then further purified separately, as describe
below.

For further purification of lysyl oxidase, the 3 M PBU
0.3 M NaCl eluate from the DEAE-Sepharose column wa
passed through a 2 litre gel-permeation column (Sephade
G25, medium grade; 5 cm × 100 cm; flow rate 40 ml/mir
previously equilibrated in PB) to quickly remove urea an
NaCl. A 400 ml column (5 cm × 20 cm) of Sephacryl S-40
was equilibrated with PB, and the protein solution in P
was loaded at 15 ml/min. (Sephacryl S-400 was used instea
of Sephacryl S-200 (Shackleton and Hulmes, 1990a), wit
no effect on recovery of enzyme activity). The column wa
washed with PB until a steady reading of absorbance a
280 nm was obtained. Finally, bound proteins were elute
with 1.5 M PBU, followed by 6 M PBU. Lysyl oxidas
appeared in the 6 M PBU eluate.

For further purification of TRAMP, the 6 M PBU/0.5 M
NaCl eluate from the DEAE-Sepharose column was usec
Urea and NaCl were removed by gel permeation and th
protein solution in PB was loaded on to Sephacryl S-400, a
above. The column was washed with PB, then bound pro
teins were eluted with 1.5 M PBU, followed by 6 M PBU
TRAMP appeared in the 1.5 M PBU eluate.

Lysyl oxidase activity was assayed by ultrafiltratio
using a ^{3}H[elastin] substrate, as described (Shackleton an
Hulmes, 1990b), with the following modifications. Prior t
the initial centrifugation step, 50% (w/v) trichloracetic aci
was added to a final concentration of 5% (w/v). From th
supernatants, 400 µl aliquots were ultrafiltered usin
Ultrafree-MC units and a Beckman JA 18.1 rotor. Fo
scintillation counting, 300 µl of the ultrafiltrate were adde
to 2.7 ml Cocktail T. Control assays contained 0.2 mM β
aminopropionitrile (BAPN), a specific inhibitor of lysy
oxidase. Where necessary, results were corrected for partia
inhibition by urea (Shackleton and Hulmes, 1990b).

Variants of both lysyl oxidase and TRAMP were sepa

ated by anion-exchange FPLC using a procedure similar to hat of Burbelo et al. (1986). Lysyl oxidase or TRAMP preparations in 6 M PBU were applied to a Pharmacia Mono Q HR 5/5 column pre-equilibrated in 6 M PBU and operated at room temperature on either a Pharmacia FPLC or Gilson HPLC system. After washing the column with 6 M PBU, bound proteins were eluted, at 1 ml/min, with a 30-ml linear gradient from 0 to 1 M NaCl in 6 M PBU.

Prior to the preparation of peptides for amino acid sequencing, solvent exchange and further purification in denaturing conditions were achieved by preparative reverse-phase FPLC. Peak fractions (approximately 1 mg) from the Mono Q column were applied to a ProRPC HR 5/2 column (Pharmacia; 5 μm particle size; 5 mm × 20 mm) pre-equilibrated in solvent A (aqueous 0.1% (v/v) trifluoroacetic acid (TFA)). Proteins were eluted at 2 ml/min with a 90 ml linear gradient of 10 to 70% solvent B (100% acetonitrile, 0.08% (v/v) TFA).

SDS-PAGE

Proteins were analysed by discontinuous gel electrophoresis (Laemmli, 1970) with 12% acrylamide, 0.32% bis-acrylamide in the separating gel. The gels were stained with Coomassie Brilliant Blue R250 (Miller and Rhodes, 1982) or Alcian Blue in the presence of 50 mM $MgCl_2$ (Wall and Gyi, 1988). Gels were scanned with a Joyce-Loebl Chromoscan 3 densitometer.

Mass determinations

Precise mass determinations were made with a laser desorption ion source coupled to a time-of-flight mass analyser (Lasermat, Finnigan MAT Ltd., Hemel Hempstead, UK). Samples (0.1–0.2 μl, approximately 6–10 pmol) were mixed with a 0.5 μl droplet of protein matrix (approximately 50 mM sinnapinic acid in 30% (v/v) 0.1% TFA, 70% acetonitrile) in the centre of the stainless steel target and the droplet was allowed to dry before introduction into the Lasermat (Karas et al., 1987). Specra from multiple laser shots were averaged to improve the signal to noise ratio. In some experiments, carbonic anhydrase (29,024 Da) was used as an internal calibrant.

Preparation of cyanogen bromide peptides

Cyanogen bromide was used to cleave proteins specifically on the C-terminal side of methionine residues (Gross and Witkop, 1962). An 175 μl aliquot of 70% (v/v) formic acid was added to 25 μl of protein solution (0.1–1.0 mg/ml). The sample was transferred to a fume cupboard, a small crystal of CNBr added, and the tube was covered in foil to exclude light. After 24 h at room temperature, water (200 μl) was added (to improve drying characteristics), then the sample was dried in a Savant Speed Vac vacuum centrifuge. To trap any HCN present, the Speedvac was connected to a chemical trap containing 2 M NaOH. The dried samples were stored at −20 °C until required for reverse-phase HPLC, at which point they were redissolved in 250 μl 6 M PBU immediately before use.

CNBr peptides were separated by HPLC on an Applied Biosystems 130 A separation system containing an Aquapore RP-300 cartridge (7 μm particle size; 2.1 × 30 mm) or, for greater resolution, an Aquapore RP-300 column (7 μm particle size; 1 mm × 250 mm). Solvents A and B were the same as for preparative reverse-phase FPLC (above). Elution conditions are shown in Fig. 4.

Amino acid analysis and sequencing

Purified peptides were sequenced using an Applied Biosystems 477 A pulsed-liquid microsequencer, as described previously (Hayes et al., 1989).

Amino acid analysis was carried out on an Applied Biosystems 420 A Derivatiser with automatic hydrolysis and an on-line Model 120 A phenylthiocarbamyl (PTC) analyser (Applied Biosystems Ltd., Warrington, Cheshire, UK). Each sample was analysed in triplicate after hydrolysis in 6 M HCl under argon for 30, 60 and 90 min at 200 °C. Values for serine and threonine were found by extrapolation to zero hydrolysis time. Values for leucine, isoleucine and valine were found from the plateau region of maximum recovery. Values for cysteine were found after vapour phase pyridylethylation of samples prior to analysis, carried out as follows. Protein samples from the reverse phase column were dried down in Pyrex tubes (6 mm × 50 mm) which were subsequently inserted into borosilicate glass vials (27 mm × 70 mm) containing 200 μl pyridine, 200 μl H_2O, 40 μl 4-vinyl pyridine and 40 μl tributylphosphine. After flushing with argon, the vials were sealed with PTFE/silicone septa, and then incubated at 60 °C for 4 h. Samples were then hydrolysed automatically (see above) or manually at 110 °C for 22 h. After manual hydrolysis, residual acid and condensation were removed in a vacuum desiccator containing pellets of NaOH, then the samples were re-dissolved in 0.025% K_3EDTA prior to amino acid analysis. The PTC-amino acids were separated at 38 °C on an Applied Biosystems PTC C-18 column (5 μm particle size; 2.1 mm × 250 mm), at a flow rate of 0.3 ml/min. The column was previously equilibrated with Buffer A (3% (v/v) acetonitrile in 50 mM sodium ethanoate, pH 5.4), and elution was by a gradient of Buffer B (70% v/v) acetonitrile in 32 mM sodium ethanoate, pH 4.7) with 2% to 64% buffer B over 18.8 min, then 64% to 100% Buffer B over 5 min. The eluate was monitored by absorbance at 254 nm.

Isoelectric point determinations

Isoelectric focussing was carried out using a BioRad Mini IEF Cell Model III. Prior to loading, samples were dialysed

into 4 M urea and adjusted to a protein concentration of approximately 1 mg/ml, using Centricon-10 microconcentrators. Samples (2 µl) were focussed on polyacrylamide gels (125 × 65 × 0.4 mm) containing 2% Ampholines (Pharmacia, UK; Ampholine mixture of pH 3.5 to 5 (5 parts) and pH 3.5 to 10 (1 part)). Protein standards of known pI were used to calibrate the pH gradients. Focussing was carried out under constant voltage conditions in a stepped fashion; 100 V for 15 min, 200 V for 15 min and finally 450 V for 1 h. When focussing was complete, proteins were stained for 2 h in 0.04% (w/v) Coomassie Brilliant Blue R250, 0.5% (w/v) $CuSO_4$, 0.05% (w/v) Crocein Scarlet 7B, 27% (v/v) ethanol, 10% (v/v) ethanoic acid. Crocein scarlet 7B was included to ensure rapid fixation of the bands, while $CuSO_4$ effectively eliminates any background staining due to the presence of Ampholines (Righetti, 1983). The gels were destained in 12% (v/v) ethanol, 7% (v/v) ethanoic acid, 0.5% (w/v) $CuSO_4$ (three changes over 45 min), followed by 25% (v/v) ethanol, 7% (v/v) ethanoic acid, until the background cleared (normally 30 min). Finally gels were air-dried in dust free conditions.

Desulphation and deglycosylation

For mild acid hydrolysis (Huttner, 1984), TRAMP variants were dialysed against distilled water, diluted with 2 M HCl to a final 1 M HCl, heated for 5 min at 100 °C, freeze dried and finally dissolved in SDS-PAGE sample buffer. For sulphatase treatment, 30 µg TRAMP were dialysed into 0.1 M sodium ethanoate (pH 5.0), 0.1 unit of sulphatase was added in a total volume of 0.2 ml, and the reaction mixture was incubated for 2 h at 37 °C. Aliquots (20 µl) were removed at set times and immediately heated for 2 min at 100 °C in SDS-PAGE sample buffer. The small amount of β-glucuronidase activity that was present as a contaminant in both sulphatases was inactivated by temporarily adjusting the pH to 2.2 (Dodgson and Spencer, 1953). Separate experiments with purified β-glucuronidase were also carried out, in identical conditions to the sulphatase treatments, at twice the levels present in the commercial sulphatase preparations.

For neuraminidase treatment, 50 µg TRAMP in 50 mM sodium ethanoate, pH 5.5, 4 mM $CaCl_2$, 100 µg/ml bovin

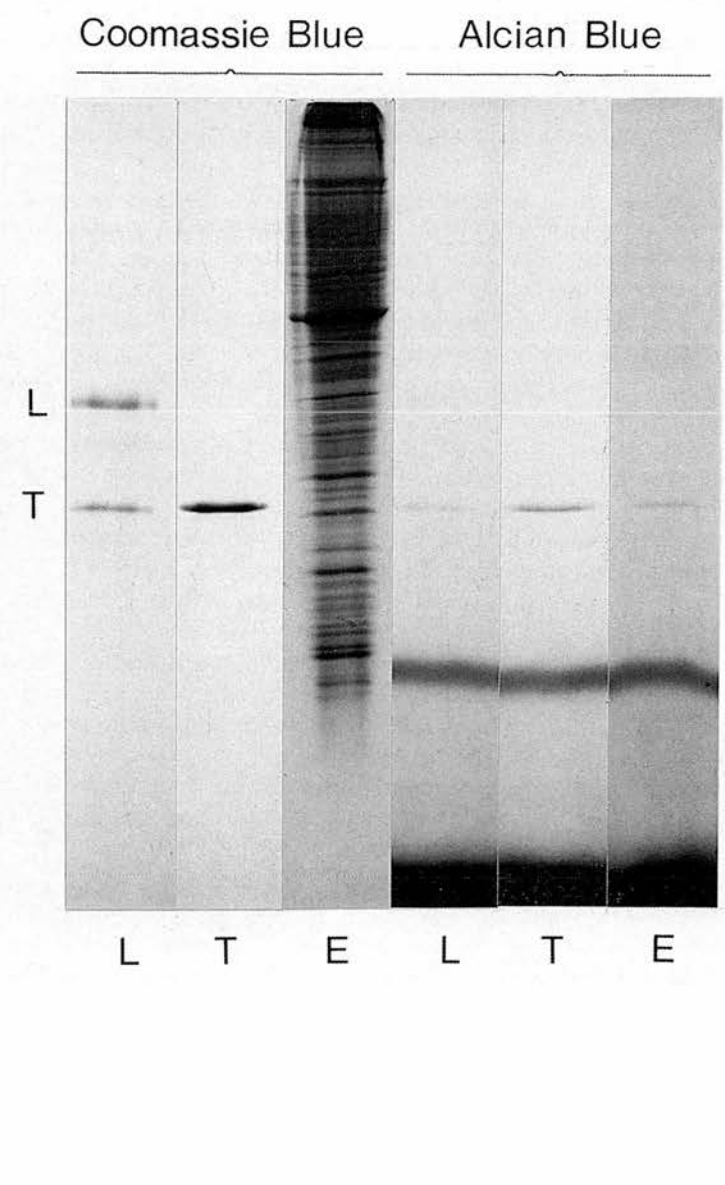


Fig. 1. SDS-PAGE of porcine skin lysyl oxidase and TRAMP partially separated by elution from DEAE-Sepharose and Sephacryl S-400 columns at different urea concentrations (see Materials and Methods). L = lysyl oxidase, T = TRAMP, E = initial 6 M PBU extract. Duplicate gels were run and equal amounts of protein were loaded in corresponding lanes for staining with either Coomassie Blue or Alcian Blue. The diffuse bands in the lower part of the Alcian Blue gel are a staining artefact.

erum albumin, 0.2 mM phenyl methyl sulphonyl fluoride PMSF), 0.2 μM N-tosyl-L-phenylalanine chloromethyl-etone (TPCK) was incubated, in a total volume of 40 μl, with 0.0008 unit neuraminidase for 18 h at 37 °C, followed by a further 6 h at 37 °C with the same amount of freshly added enzyme. The reaction was stopped by addition of SDS-PAGE sample buffer and heating for 3 min at 100 °C.

For endoglycosidase F/N-glycosidase F treatment, 50 μg TRAMP was first denatured by heating at 100 °C for 3 min in the presence of 10 mg/ml SDS. To 10 μl, 90 μl of digestion buffer (0.25 M sodium ethanoate, 20 mM EDTA, 10 mM 2-mercaptoethanol, 6 mg/ml Nonidet P-40, 5 mg/ml CHAPS, 0.2 mM PMSF, 0.2 μM TPCK, pH 6.25) was added, followed by 7.2 μl endoglycanase F/N-glycosidase F (0.36 unit), and the mixture was incubated at 37 °C for 18 h. Incubations were also carried out at pH 4.5 and pH 7.5, to favour the activities of endoglycosidase F and N-glycosidase F, respectively. Reactions were stopped by addition of SDS-PAGE sample buffer and heating for 3 min at 100 °C.

For treatment with O-glycosidase, 200 μg protein (in 100 μl of 20 mM sodium cacodylate buffer pH 6.0) was incubated in the presence of 0.001 unit enzyme for 16 h at 37 °C and the reaction was stopped by addition of SDS-PAGE sample buffer and heating for 2 min at 100 °C, followed directly by electrophoresis or storage at −30 °C. Fetuin was used as a positive control, and as the presence of sialic acid interferes with O-glycosidase activity, incubations with O-glycosidase were done both with and without prior neuraminidase treatment.

Results

Identification of lysyl oxidase and TRAMP variants

Lysyl oxidase and TRAMP were almost completely separated when the DEAE-Sepharose and Sephacryl S-400 columns were eluted at different urea concentrations, as shown by SDS-PAGE (Fig. 1). Lysyl oxidase eluted from DEAE-Sepharose in the presence of 0.3 M NaCl at a relatively low concentration of urea, while a relatively high urea concentration was required to elute the enzyme from Sephacryl S-400. The converse was true of TRAMP. After further purification by anion-exchange Mono Q FPLC, both proteins eluted in the form of several peaks, with some overlap between individual lysyl oxidase and TRAMP variants (Fig. 2). Peaks from the Mono Q column were analysed by SDS-PAGE (not shown), and the electrophoretic migration of the four lysyl oxidase variants (Fig. 2(a)) was identical. Similarly, all four variants of lysyl oxidase eluted at the same position by reverse phase FPLC (e.g. Fig. 3). By the same criteria, all five variants of TRAMP (Fig. 2(b)) were identical, but their electrophoretic migration and reverse-phase chromatographic elution positions were distinct from lysyl oxidase. No lysyl oxidase activity was associated with TRAMP.

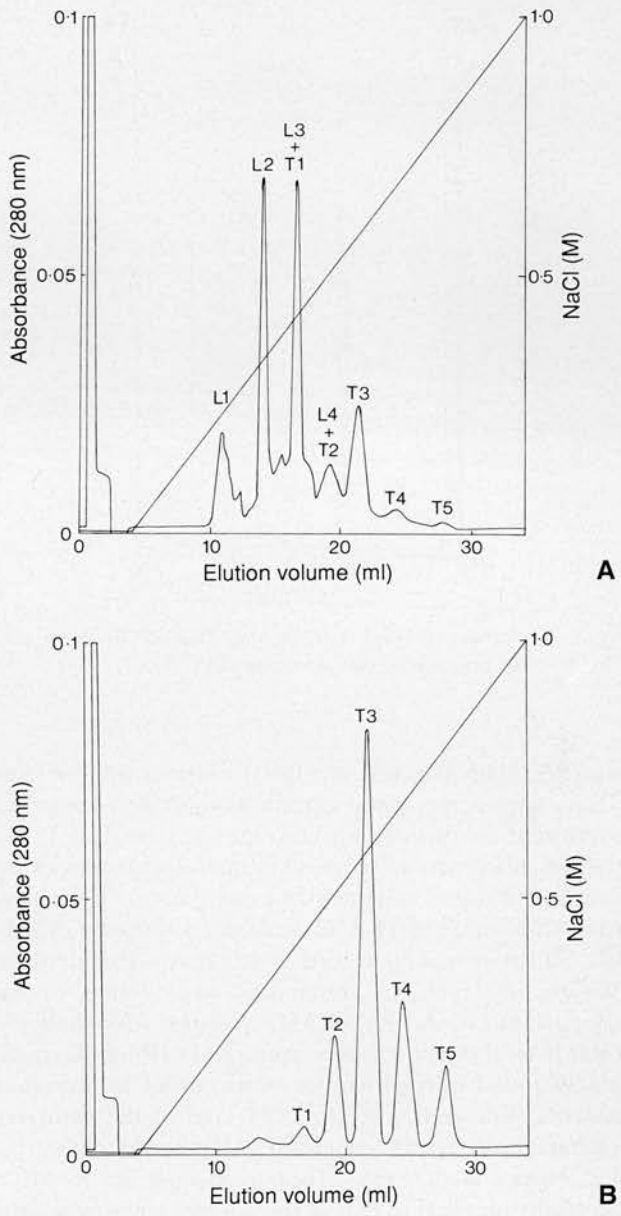


Fig. 2. Separation of lysyl oxidase and TRAMP variants by Mono Q FPLC, following partial purification by DEAE-Sepharose and Sephacryl S400 chromatography. (a) lysyl oxidase variants L1 to L4, with small amounts of TRAMP variants T1 to T5 (b) TRAMP variants T1 to T5.

Peptide analysis and sequencing

Variants of lysyl oxidase and TRAMP were subjected to amino acid analysis. The amino acid composition of porcine skin lysyl oxidase (variant L3) is shown in Table I. The composition is very similar to that of the rat aorta (Trackman et al., 1990; Trackman et al., 1991) and human

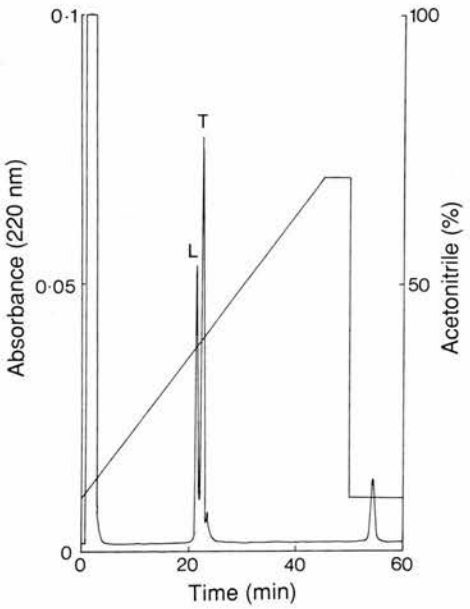


Fig. 3. Separation of lysyl oxidase and TRAMP in denaturing conditions by preparative reverse-phase FPLC.

placenta (Hamalainen et al., 1991) enzymes derived from cDNA sequencing, given certain assumptions about the position of the pro-enzyme cleavage site (see Table I). No obvious differences were found in the compositions of the four lysyl oxidase variants (data not shown). The amino acid composition of TRAMP variant T3 is shown in Table II. The most striking feature is the relative abundance of tyrosine. No obvious differences were found in the compositions of the five TRAMP variants. Also shown in Table II are the amino acid compositions of the 23 K contaminant found in lysyl oxidase preparations from human placenta (Kuivaniemi et al., 1984) and of the dermatan sulphate proteoglycan-associated 22 K protein from bovine skin (Neame et al., 1989). The composition of TRAMP is essentially identical to that of the bovine skin protein, and there are some similarities with the human placental 23 K protein of Kuivaniemi et al. (1984).

Variants of lysyl oxidase were treated with cyanogen bromide and the resulting peptides separated by reverse-phase HPLC. The elution profile for variant L3 is shown in Fig. 4(a). All the lysyl oxidase variants gave similar CNBr profiles (data not shown). Variants of TRAMP were also treated with CNBr, and the HPLC analysis of peptides from variant T3 is shown in Figs. 4(b) and 4(c). Similar CNBr profiles for the other TRAMP variants were obtained (data not shown). There were no similarities between the CNBr profiles of lysyl oxidase and TRAMP.

Fig. 5 shows amino acid sequence data for selected CNBr peptides of lysyl oxidase and TRAMP. The sequence of the 36 residue peptide from porcine skin lysyl oxidase is identical to residues 291 to 326 in the rat aorta lysyl oxidase

Table I. Amino acid composition of lysyl oxidase from various species.

Amino acid	Porcine skin[a]	Rat aorta[b]	Human placenta[c]
D	28.7	32	33
E	18.0	19	17
S	22.2	23	18
G	14.0	13	15
H	12.7	12	11
R	19.1	18	18
T	11.6	12	14
A	16.0	15	15
P	21.6	16	16
Y	26.1	30	31
V	12.7	13	12
M	3.0	3	3
C	6.4	11	11
I	7.5	7	7
L	16.1	13	14
F	6.9	6	6
K	4.0	5	6
W	N.D.	3	4
Totals	246.6	251	251

[a] Variant L3 (see Fig. 2(a)) Values are residues per molecule, normalised on the assumption that the mature form of lysyl oxidase contains 16-alanine residues (see notes b and c). ND = not determined.

[b] From cDNA sequencing of the rat lysyl oxidase precursor (Trackman et al., 1990; Trackman et al., 1991), assuming the mature enzyme consists of the entire C-terminal region beginning at residue 161 (based on preliminary observations on porcine skin lysyl oxidase by mass spectrometry (Cronshaw, Shackleton, Hulmes and Fothergill-Gilmore, unpublished observations).

[c] From cDNA sequencing of the human lysyl oxidase precursor (Hamalainen et al., 1991), assuming the mature enzyme consists of the entire C-terminal region beginning at residue 167 (see note b).

precursor (Trackman et al., 1990; Trackman et al., 1991) and it differs in only four positions from residues 299 to 334 of the human placental enzyme precursor (Hamalainen et al., 1991). The amino acid sequence of the 44-residue TRAMP peptide was not found in lysyl oxidase. However, when compared with the Protein Identification Resource database (release 30) using the program 'prosrch' (Coulson et al., 1987), the TRAMP sequence was found in the 22 K dermatan sulphate proteoglycan-associated extracellular matrix protein from bovine skin (Neame et al., 1989). The sequence from TRAMP is identical to residues 83 to 126 of the bovine skin protein, and this degree of matching is highly significant (Collins et al., 1988). From the similarities in sequence and molecular mass, we conclude that TRAMP is the same as the protein of Neame et al. (1989).

Table II. Amino acid composition of TRAMP and related proteins.

Amino acid	Porcine skin[a]	Bovine skin[b] (22K protein)	Human placenta[c] (Contaminant, pool II)
D	19.6	20	14.1
E	23.6	24	21.9
S	11.9	13	10.5
G	13.1	13	14.3
H	2.9	4	2.5
R	12.3	12	9.0
T	7.4	9	6.8
A	7.0	7	7.0
P	6.7	6	5.9
Y	18.1	20	8.4
V	8.0	9	6.4
M	6.8	7	ND
C	9.2	10	ND
	3.8	4	4.1
	3.7	5	4.1
	7.7	8	5.4
K	4.0	4	4.8
W	ND	8	ND

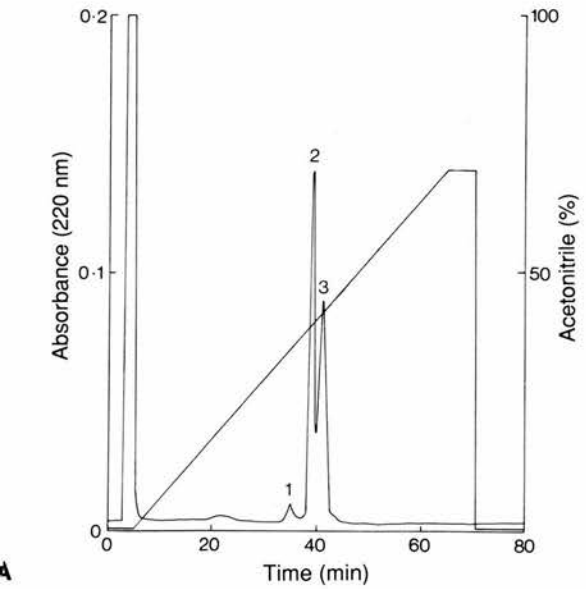
ND = not determined.

[a] Variant T3 (see Fig. 2(b)). Values are residues per molecule, normalised to 7 alanine residues (from Neame et al., 1989; Cronshaw, Hulmes and Fothergill-Gilmore, manuscript in preparation).

[b] From protein sequence data of Neame et al. (1989), expressed as residues per molecule.

[c] From Kuivaniemi et al. (1984), re-expressed as residues per molecule, assuming 7 alanine residues.

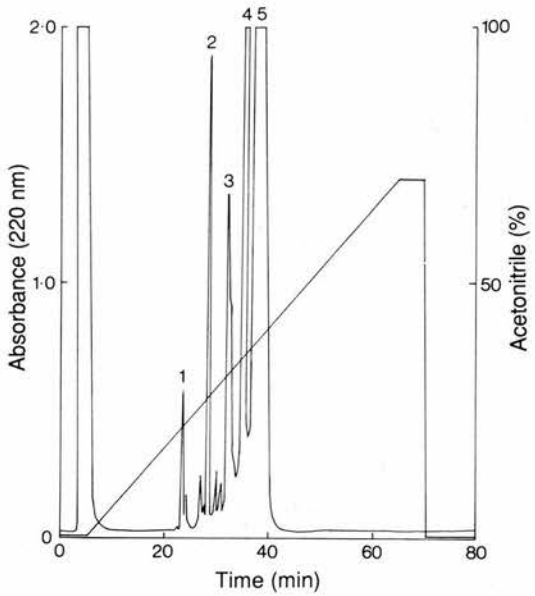
Fig. 4. Reverse phase HPLC elution profiles of CNBr peptides. (a) Lysyl oxidase variant L3 (b) TRAMP variant T3, non-reduced (c) Peak 5 in (b) after reduction with 0.1 M dithiothreitol.



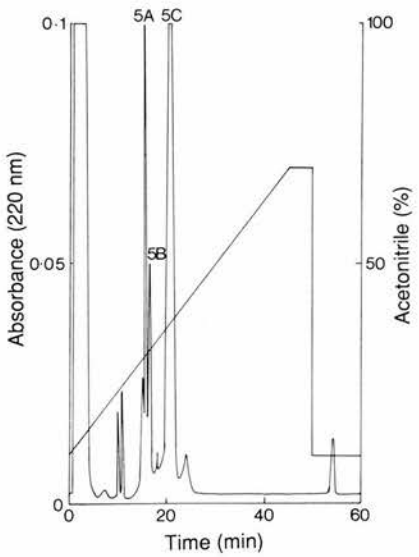
A

Further characterisation of TRAMP

The amino acid sequence of TRAMP (Neame et al., 1989) is rich in acidic amino acids. From complete sequence data for porcine TRAMP (Cronshaw, Hulmes and Fothergill-Gilmore, manuscript in preparation) the calculated isoelectric point (pI; determined with the University of Wisconsin GCG software package, and allowing for pyroglutamate at the N-terminus) is 4.53. To determine the pI experimentally, TRAMP variants were analysed by isoelectric focussing in the presence of 4 M urea. The results (Table III) show that TRAMP variants T1 to T5 are increasingly acidic, with pI's in the pH range 4.43 to 4.07. The observed pI's are less than the theoretical value, and they decrease as expected from the Mono Q elution profile



B



C

(Fig. 2). Similar observations were made by chromatofocussing (data not shown).

More precise molecular mass determinations of TRAMP variants were obtained by mass spectrometry. Molecular masses for all TRAMP variants are shown in Table III. Based on the complete amino acid sequence of porcine TRAMP (Cronshaw, Hulmes and Fothergill-Gilmore, manuscript in preparation), the calculated relative molecular mass in non-reducing conditions is 21989. The observed relative molecular masses of all TRAMP variants are greater than the calculated value, and the masses increase monotonically with decreasing isoelectric point (Table III).

The differences in the observed and calculated molecular masses and isoelectric points suggest that some amino acid residues in TRAMP may be modified at the post-translational level. The modification(s) would be acid labile, to account for the apparent absence of modified amino acids by sequence analysis, and would increase the net negative charge, to account for the low pI. The presence of an acidic post-translational modification was suggested by the observation that TRAMP stains strongly with Alcian Blue (Fig. 1). Following SDS-PAGE, a protein with the electrophoretic migration of TRAMP was the only one to stain with Alcian Blue in the original 6 M urea extract (Fig. 1). On the basis of the Alcian Blue staining is was possible to estimate that 1 g of piglet skin (wet weight) contains approximately 4 μg of TRAMP.

Possible glycosylation of TRAMP was investigated as follows. By amino acid analysis, there was no evidence for hexosamines in TRAMP, as also observed by Neame et al. (1989). Furthermore, after digestion of TRAMP variants with endoglycosidase F/N-glycosidase F, neuraminidase, O-glycosidase or β-glucuronidase, there were no changes in electrophoretic mobility or band sharpness by SDS-PAGE (not shown) or in Alcian Blue staining relative to Coomassie Blue staining (Fig. 6).

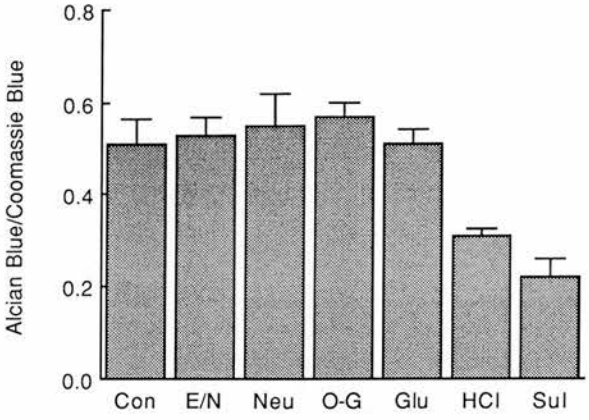


Fig. 6. Effects of various deglycosylation and desulphation treatments on TRAMP staining by Alcian Blue. Staining of SDS-PAGE gels was quantitated with a scanning densitometer and expressed relative to Coomassie Blue staining of equal amounts of protein treated in identical conditions. Error bars are standard deviations based on at least three measurements. TRAMP was analysed after purification by DEAE-Sepharose and Sephacryl S400 chromatography, without subsequent separation of ionic variants. Con = control; E/N = endoglycosidase F/N-glycosidase F; Neu = neuraminidase; O−G = O-glycosidase; Glu = β-glucuronidase; HCl = 1 M HCl; Sul = sulphatase (type V).

(a) Lysyl oxidase (L3, CNBr peptide 1)

```
          1         5         10        15        20
          |         |         |         |         |
porcine   D E F S H Y D L L D A S T Q R R V A E G
human     - - - - - L Y - - - - N - - - - W - - -

                  25        30        35
                  |         |         |
porcine   H K A S F C L E D T S C D Y G Y
human     - - - - - - - - - - - - - - - -
```

(b) TRAMP (T3, CNBr peptide 5C)

```
1         5         10        15        20
|         |         |         |         |
E W Y Q T C S N N G L V A G F Q S R Y F

          25        30        35        40
          |         |         |         |
E S V L D R E W Q F Y C C R Y S K R C P

      44
      |
Y S C W
```

Fig. 5. Amino acid sequences of CNBr peptides. (a) Lysyl oxidase variant L3, peak 1 from Fig. 4(a). (b) TRAMP variant T3, peak 5C from Fig. 4(c). The sequence of the peptide from lysyl oxidase is identical to residues 291 to 326 of the rat enzyme precursor (Trackman et al., 1990; 1991). Differences from residues 299 to 334 of the human pro-enzyme (Hamalainen et al., 1991) are indicated. The sequence of the TRAMP peptide is identical to residues 83 to 126 of the 22 K protein from bovine skin (Neame et al., 1989). Initial yields and repetitive yields were 100 pmol and 91% (based on phenylalanine) for the lysyl oxidase peptide, and 850 pmol and 90% (based on valine) for the TRAMP peptide.

Table III. Molecular masses and isoelectric points (pI) of TRAMP variants.

Variant	T1	T2	T3	T4	T5
Mass[a] (Da)	22119 ± 22	22183 ± 22	22252 ± 22	22306 ± 22	22352 ± 22
pI[b]	4.43 ± 0.1	4.34 ± 0.06	4.27 ± 0.08	4.18 ± 0.08	4.07 ± 0.06

[a] Errors are standard deviations based on standards (carbonic anhydrase, n = 8).
[b] Errors are standard deviations (n = 6).

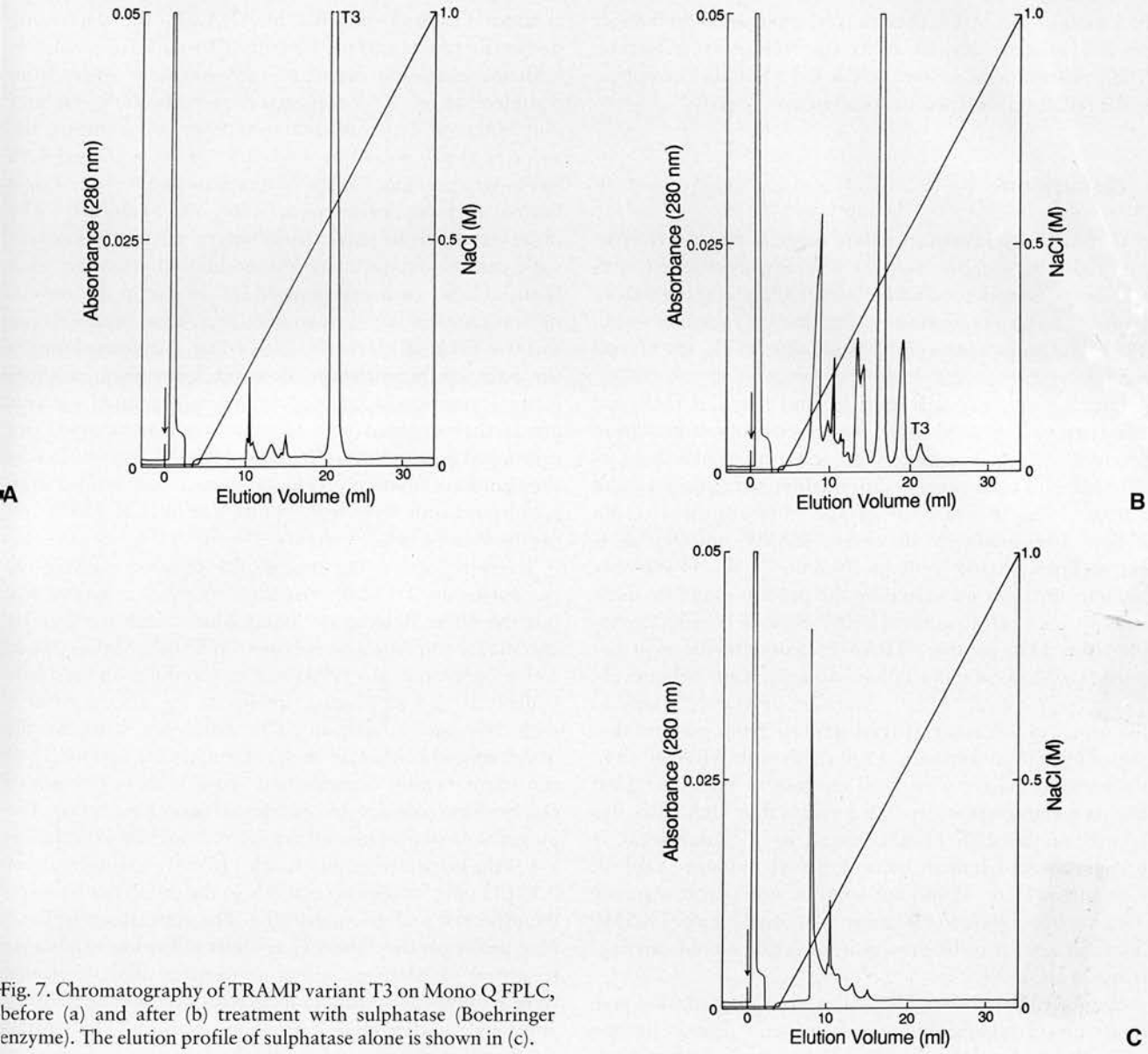


Fig. 7. Chromatography of TRAMP variant T3 on Mono Q FPLC, before (a) and after (b) treatment with sulphatase (Boehringer enzyme). The elution profile of sulphatase alone is shown in (c).

One possible post-translational modification is tyrosine sulphation. There are five tyrosine residues in TRAMP that meet four of the five empirically determined rules for tyrosine sulphation (Hortin et al., 1986) involving the proximity of acidic residues and relative paucity of basic residues, hydrophobic residues and cysteine. These putative tyrosine sulphation sites are residues 5, 15, 148, 149 and 176 in the sequence of Neame et al. (1989). The tyrosine sulphate ester bond is particularly labile to acid hydrolysis (Huttner, 1984). Following treatment of TRAMP with 1 M HCl for 5 min at 100 °C, Alcian Blue staining almost halved relative to Coomasie Blue staining (Fig. 6), though there was evidence of partial hydrolysis (not shown). To further investigate possible tyrosine sulphation, TRAMP was incubated with sulphatase (either type V or type VII) prior to SDS-PAGE. Following treatment with either enzyme, Alcian Blue staining relative to Coomassie Blue staining decreased almost 3-fold (Fig. 6), with no apparent changes in electrophoretic mobility or band sharpness (not shown). In still further experiments, the chromatographic behaviour of the T3 variant was examined on Mono Q FPLC before and after sulphatase treatment. The results (Fig. 7) show that while a small protein peak continued to be present after sulphatase treatment at the original elution position of T3, three additional peaks appeared earlier in the elution gradient. When examined by SDS-PAGE (not shown), all three peaks co-migrated with T3.

As TRAMP co-purifies with lysyl oxidase, possible interactions between these proteins were examined. We found that increasing concentrations of TRAMP, up to a 40

to 1 molar ratio of TRAMP to lysyl oxidase, had no effect on lysyl oxidase activity using the ^{3}H[elastin] substrate. These observations do not exclude the possibility of a physical interaction with no effect on enzyme activity.

Discussion

Our amino acid sequence data confirm that the enzyme purified from porcine skin by selective interaction with Sephacryl (Shackleton and Hulmes, 1990a) corresponds to both rat aorta (Trackman et al., 1990; Trackman et al., 1991) and human placental (Hamalainen et al., 1991) lysyl oxidase.

Initially we were surprised to find that TRAMP was unrelated to lysyl oxidase, as the presence of a degradation product with approximately the same molecular mass as TRAMP had been suggested in the literature (Sullivan and Kagan, 1982; Kuivaniemi et al., 1984; Burbelo et al., 1986). The similarity between TRAMP and the 22 K extracellular matrix protein (Neame et al., 1989) only became apparent on searching the protein sequence database. By urea extraction and DEAE-Sephacel chromatography, the 22 K protein (TRAMP) from bovine skin co-purifies with dermatan sulphate proteoglycans (Choi et al., 1989; Neame et al., 1989). A similar procedure is used in the initial purification of lysyl oxidase from porcine skin (Shackleton and Hulmes, 1990a), though TRAMP continues to co-purify with lysyl oxidase in the subsequent Sephacryl interaction step. It is possible that TRAMP is also related to the 23 K protein found as a contaminant in preparations of human placental lysyl oxidase (Table II; Kuivaniemi et al., 1984), but without amino acid sequence data it is impossible to make any firm conclusions. TRAMP does not appear to be present in urea extracts of cartilage (Choi et al., 1989).

We have shown that TRAMP is not a degradation product of lysyl oxidase. There is, however, a distinct protein of similar molecular mass (22–24 K) which, from peptide mapping (Sullivan and Kagan, 1982) or immunoblotting (Kuivaniemi et al., 1984; Burbelo et al., 1986), does appear to be a genuine degradation product of the enzyme.

It is not clear why both lysyl oxidase and TRAMP should exist in several variant forms. The presence of multiple, ionic variants of lysyl oxidase has also been observed in enzyme preparations from chick cartilage (Stassen, 1976), bovine aorta (Sullivan and Kagan, 1982) and human placenta (Kuivaniemi et al., 1984). Only one gene for lysyl oxidase has been found in rat genomic DNA (Trackman et al., 1990), so post-translational modifications are a likely cause of the variation. Another possibility is carbamylation at lysine residues while proteins are in 6 M urea (Means and Feeney, 1971); both TRAMP (Neame et al., 1989) and lysyl oxidase (Trackman et al., 1990; Trackman et al., 1991; Hamalainen et al., 1991) contain small numbers of lysine residues (Tables I and II). In TRAMP, the decreasing isoelectric points (pI) for variants T1 to T5 are consistent with an increasing extent of carbamylation since, from complete amino acid sequence data (Cronshaw, Hulmes and Fothergill-Gilmore, manuscript in preparation), the theoretical pI's would be 4.53, 4.44, 4.36, 4.28 and 4.20 (with pyroglutamate at the N-terminus and 0, 1, 2, 3 or 4 lysines carbamylated, respectively; cf. (Table III). The observations from mass spectrometry are also consistent with carbamylation, since the additional mass for each lysine residue carbamylated (43 Da) is within the experimental range of the average mass increment between consecutive TRAMP variants (32 to 76 Da). If carbamylation is the basis of the variation, however, conversion to more acidic forms would be expected when separated variants are further exposed to 6 M urea. We have carried out prolonged incubations of purified TRAMP variants in 6 M urea but have found no evidence for such conversion. Similar observations were reported by Kagan et al. (1979) on purified lysyl oxidase variants.

Several observations suggest the presence of tyrosine sulphation in TRAMP. Sequence analysis indicates five possible sites. Staining by Alcian Blue, which is relatively specific for sulphated biopolymers in 50 mM $MgCl_2$ (Scott, 1973; Newton et al., 1974) and susceptibility to mild acid hydrolysis and sulphatase treatment are also consistent with tyrosine sulphation. The difference between the observed and calculated molecular mass for variant T1 is consistent, within experimental error, with sulphation of two tyrosine residues (i.e. additional mass 2×79 Da). The presence of two sulphated tyrosines would be expected to lower the isoelectric point of each TRAMP variant by about 0.17 pH unit, which corresponds to the difference between the observed and calculated pI's. The appearance of three new peaks on the Mono Q column following sulphatase treatment of T3 suggests that up to three sulphate groups were removed, resulting in a corresponding increase in pI and hence earlier elution from the column. The elution positions of the two peaks to the left of T3 (following sulphatase treatment) correspond to those of the T1 and T2 variants (without sulphatase treatment) but we cannot conclude from this that variants T1 to T5 differ in their extent of sulphation. It may be that each isoform is sulphated to the same extent and the variation arises from an additional modification (e.g. carbamylation) in which case each variant would generate up to three products on sulphatase treatment. Experiments are in progress to examine these possibilities. Taken together however, these observations are consistent with, but do not establish, the presence of tyrosine sulphation in TRAMP. Tyrosine sulphation is, however, a widespread post-translational modification in extracellular matrix proteins, e.g. nidogen/entactin (Paulsson et al., 1985), procollagen V (Fessler et al., 1986), procollagen III (Jukkola et al., 1986), bone sialoprotein II (Ecarot-Charrier et al., 1989; Midura et al., 1990), fibro-

nectin (Liu et al., 1985), dermatan sulphate proteoglycan (Huttner, 1988) and fibromodulin (Antonsson et al., 1991). The site of tyrosine sulphation in fibromodulin is in an N-terminal tyrosine-rich region in which tyrosine is every second or third residue (Antonsson et al., 1991). This feature is also present in TRAMP (Neame et al., 1989), though the sequences are not homologous. Multiple variants of fibromodulin have also been observed (Lauder, R.M., Nieduszynski, I.A. and Huckerby, T.N., personal communication). The role of tyrosine sulphation in extracellular matrix proteins has not been extensively investigated, though desulphation of fibronectin affects binding affinities to several ligands (Suiko and Liu, 1988). The absence of staining by Alcian Blue (Fig. 1) suggests that lysyl oxidase is not sulphated.

The function of TRAMP is not well understood. A possible role in cell adhesion has been suggested for the equivalent 22 K protein from bovine skin (Lewandowska et al., 1991). We have found that TRAMP has no lysyl oxidase activity on an elastin substrate, nor does it affect the activity of added lysyl oxidase. This lack of interaction with elastin is consistent with the apparent absence of TRAMP in lysyl oxidase when purified from rat skin by an elastin affinity method (Romero-Chapman et al., 1991). We have observed, however, that TRAMP binds to collagen and accelerates fibril formation *in vitro* (MacBeath, Shackleton and Hulmes, unpublished observations). As it is known that lysyl oxidase activity on collagen increases following fibril formation (Siegel, 1979), it is possible that TRAMP may influence lysyl oxidase activity indirectly, via aggregation of the substrate. The observations that the bovine skin 22 K protein interacts with dermatan sulphate proteoglycan (DSPG; Choi et al., 1989; Neame et al., 1989; Lewandowska et al., 1991) and that DSPG II (decorin) is known to inhibit collagen fibril formation (Brown and Vogel, 1989) suggest a complex regulatory role for TRAMP in interactions with other extracellular matrix components.

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